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TITLE: Molecular Epidemiology Investigation of Obesity and Lethal Prostate Cancer

PRINCIPAL INVESTIGATOR: Ericka Ebot (Noonan)

CONTRACTING ORGANIZATION:
Harvard T. H. Chan School of Public Health
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14. ABSTRACT

The major objective of the PCRP postdoctoral training award was to provide training and opportunities for the principal investigator (PI) to further her development as an independent prostate cancer researcher in the field of molecular epidemiology. During the award period the PI performed research incorporating tissue-level biomarker data into epidemiologic studies of prostate cancer progression to investigate the relationship between obesity, the tumor microenvironment, and lethal prostate cancer. Using whole transcriptome gene expression profiling data, she identified several gene signature enriched in the tumor tissue of overweight and obese prostate cancer patients. Furthermore, several of these signatures were associated with poor prognosis. The findings supported an epigenetic link between obesity and prostate cancer survival which will be explored in future studies. The support of the award has provided many opportunities to enhance the professional development of the PI. The coursework and research activities accomplished over the past two years have strengthened her research skills. The PI has presented her research at a number of meetings and conferences and has submitted a manuscript of the findings from this award. During this period she has developed new collaborations that have led to exciting new research opportunities.

15. SUBJECT TERMS
Lethal prostate cancer, obesity, tissue biomarkers, gene expression, growth factor signaling, inflammation, angiogenesis, molecular epidemiology

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INTRODUCTION

A significant challenge in prostate cancer research is the identification of factors that drive disease progression. Obesity is a particularly compelling risk factor for lethal disease due to its high prevalence in the United States and its potential as a modifiable risk factor. In the United States, one-third of men are obese and another one third are overweight[1]. While not related to overall prostate cancer risk, obesity is strongly linked with risk of advanced disease and worse cancer-specific outcomes[2-4]. However, what drives the association between obesity and lethal prostate cancer is not well understood. Obesity dysregulates multiple hormonal and metabolic pathways and is associated with higher levels of insulin and insulin-like growth factor-1 (IGF-1), lowers level of adiponectin, lower levels of testosterone, and higher levels of inflammatory cytokines, all of which may be factors in prostate progression through direct effects on the tumor microenvironment[5]. In this proposal we seek to develop a better understanding of the link between obesity and lethal disease, in order to improve our ability to develop successful interventions strategies and therapies for men diagnosed with prostate cancer.

KEYWORDS

Lethal prostate cancer, obesity, tissue biomarkers, gene expression, growth factor signaling, inflammation, angiogenesis, molecular epidemiology

ACCOMPLISHMENTS

What were the major goals of the project?

Mentored Training Plan

The goal of the mentored training plan was to provide training and opportunities for the principle investigator (PI) that will promote her career development as an independent prostate cancer researcher in the field of molecular epidemiology. The following tasks were proposed: 1) meet with mentor and collaborators to discuss research progress, 2) attend seminars and present research results at prostate cancer meetings at HSPH and the Dana Farber/Harvard Cancer Center (DF/HCC), 3) complete coursework in advanced biostatistics methods, epidemiology study design, biomarker and pathology techniques for epidemiology studies, and clinical research strategies, and 4) attend scientific conferences to disseminate research findings.

Research Project

The objective of the research project was to quantify the link between the prostate tumor microenvironment and prostate cancer mortality, with a focus on obesity as a driver of lethal prostate cancer. The proposal focused on three key obesity-related biological processes including growth factor signaling, inflammation, and angiogenesis, and highlighted the integration of tissue biomarker data with anthropometric and cancer outcome data to elucidate the relationship between obesity and lethal disease. The research project aimed to 1) evaluate the association between obesity and markers of growth factor signaling, inflammation, and angiogenesis in the tumor microenvironment, 2) define the link between obesity, markers of growth factor signaling, inflammation, and angiogenesis in the tumor microenvironment and lethal disease, and 3) perform a discovery analysis on the association between obesity and lethal prostate cancer using gene expression data.

What was accomplished under these goals?

Mentored Training Plan

Task 1: Meet with mentor and collaborators

The PI consulted regularly with her primary mentor (Lorelei Mucci) as well as other key collaborators on the project (Stephen Finn, Svitlana Tyekucheva, Christopher Sweeney).

Task 2: Attend seminars and meetings

The PI regularly attended weekly Epidemiology Seminars offered by the Department of Epidemiology at HSPH and Bioinformatics/Omics Seminars offered by the Channing Division of Network Medicine (CDNM) at Brigham and Women's Hospital. In addition, she attended and presented research progress at various meetings throughout the award period including Patho-epidemiology Group meetings, Prostate Cancer Epidemiology Group meetings, DF/HCC SPORE in Prostate Cancer meetings, and ToPCaP (Transdiciplinary Prostate Cancer Partnership) conference calls. Dr. Ebot was selected for a travel award to present her research findings

at the Annual Prostate Cancer SPORE Retreat in Fort Lauderdale, FL in March 2015 (see annual progress report Appendix 1). In addition, she was asked to present at the Annual ToPCaP Retreat in Ireland in September 2015 (see Appendix 1). Furthermore, she had the opportunity to present research findings at a number of meetings in and around HSPH including the DF/HCC Celebration of Junior Investigators in Cancer Science (see annual progress report Appendix 2), the HSPH Program in Genetic Epidemiology and Statistical Genetics Seminar Series (see annual progress report Appendix 3), the Meeting on Lipid Metabolism and Metabolic Alterations in Prostate Cancer at Dana-Farber Cancer Institute (see annual progress report Appendix 4), the Patho-epidemiology Mini Retreat at HSPH (see Appendix 2), and the CDNM Tissue Working Group at Brigham and Women's Hospital (see Appendix 3). Details of the presentations are provided below (see Publications, conference papers, and presentations).

Task 3: Complete Coursework
Dr. Ebot completed the Harvard Catalyst course Applications in Network Medicine: Gene Co-expression and Gene Regulatory Networks in fall 2015. In spring 2016, she completed the Harvard Catalyst course Understanding Biomarker Science: From Molecules to Images. In addition, she had the opportunity to shadow Dr. Sweeney to gain a first-hand look at patient care by a medical oncologist.

Task 4: Attend scientific conferences
The PI attended the AACR Annual Meeting 2015 in Philadelphia, PA in April 2015. Her abstract was chosen for a talk in the Molecular and Genetic Epidemiology of Cancer 4: New Insights Minisymposium (see annual progress report Appendix 5). She was awarded a Scholar-in-Training Award from the AACR Molecular Epidemiology Work Group to attend this meeting. As a result of this funding she was also able to attend and present a poster at the AACR Metabolism and Cancer Conference in Bellevue, WA in June 2015 (see annual progress report Appendix 6). Details of the presentations are provided below (see Publications, conference papers, and presentations).

Research Project
Aim 1: Evaluate the association between obesity and markers of growth factor signaling, inflammation, and angiogenesis in the tumor microenvironment
We examined the association between obesity measures (body mass index (BMI) and waist circumference) at diagnosis and immunohistochemistry (IHC) markers of insulin/IGF-1 signaling (insulin receptor, IGF-1 receptor, PTEN, pAKT, pS6 and stathmin), histologic measures of acute and chronic inflammation, and histologic measures of microvessel density and morphology in prostate tumor tissue. No significant associations were identified for the insulin/IGF-1 signaling markers or for the microvessel density and morphology measures. We identified a positive association between BMI at diagnosis and severity of chronic inflammation in tumor tissue; however the statistical significance was borderline (Table 1, unpublished data).

Table 1. Association between obesity measures and presence of acute and chronic inflammation

		BMI (kg/m^2)		Waist Circumference (inches)	
	No.	Average	P-value	Average	P-value
Acute inflammation					
No	657	25.8	0.822	38.1	0.352
Yes	242	25.9		38.4	
Chronic inflammation					
No	125	25.4	0.045	37.6	0.143
Mild	448	25.8		38.2	
Moderate	253	26.0		38.0	
Severe	73	26.4		39.1	

P-values from t-test for acute inflammation and linear regression for chronic inflammation

Aim 2: Define the link between obesity, markers of growth factor signaling, inflammation, and angiogenesis in the tumor microenvironment and lethal disease

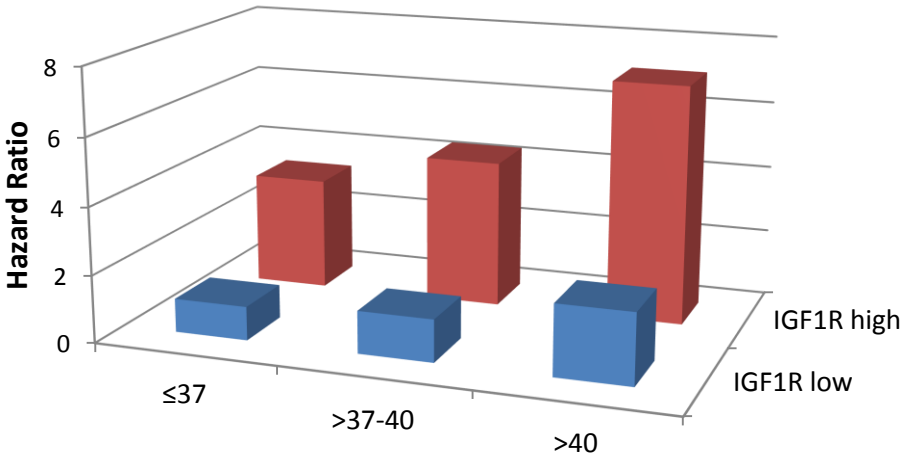
Aim 2a: We evaluated the association between the tissue-level markers described in Aim 1 and lethal prostate cancer overall and by obesity status. Specifically, we were interested in whether any of these biological factors modified the association between obesity measures (BMI and waist circumference) at diagnosis and lethal prostate cancer. Insulin/IGF-1 signaling: We did not identify any significant interactions for the insulin receptor, PTEN, pAKT, pS6, or stathmin. We did note, however, that the effect of waist circumference on prostate cancer survival after diagnosis is greater among men with tumors expressing IGF-1R compared to those not expressing the receptor (Table 2, unpublished data). The hazard ratio (95% CI) for lethal prostate cancer among men with low IGF-1R tumor expression was 0.90 (0.33, 2.45) per 8 inch increase in waist circumference compared to 3.37 (1.17, 9.7) among men with high IGF-1R tumor expression (interaction p-value = 0.072). These results were not observed when using BMI as the obesity measure. Figure 1 illustrates the hazard ratios for lethal prostate cancer according to cross-classified categories of waist circumference and IGF-1 receptor tumor status. Men with high IGF-1 receptor status and high waist circumference at diagnosis are at a seven fold greater risk of dying from prostate cancer compared to those with low receptor expression and healthy waist circumference. Inflammation: No significant interactions were observed between BMI or waist circumference and measures of acute and chronic inflammation. Angiogenesis: No significant interactions were observed between BMI or waist circumference and measures of microvessel density and morphology. Aim 2b: We confirmed using a mediation statistical analysis that none of the tissue-level markers described above are mediators of the association between obesity and lethal disease in our data.

Table 2. Hazard ratios (95% CIs) for the association between obesity and lethal prostate cancer according to IGF-1 receptor tumor status

	IGF1R low [0,2]			IGF1R high [2.17,3]			
	#	N	HR (95% CI)	#	N	HR (95% CI)	P inter
Body mass index							
Continuous (per 5 kg/m^2)	33	486	1.27 (0.75, 2.16)	22	182	1.60 (0.82, 3.14)	0.533
Waist circumference							
Continuous (per 8 inches)	22	402	0.90 (0.33, 2.45)	20	143	3.37 (1.17, 9.7)	0.072

Cox proportional hazards regression model adjusted for age and year of diagnosis

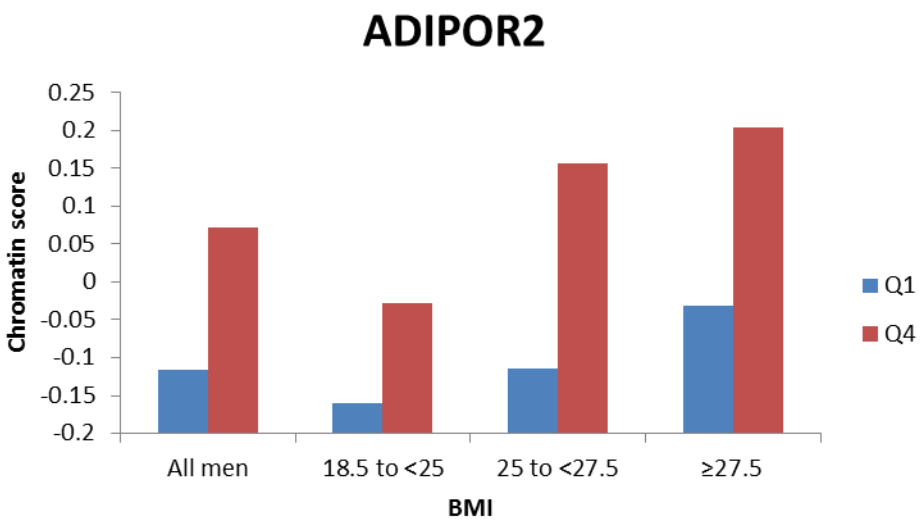
Figure 1. Hazard ratios for lethal prostate cancer by cross-classified categories of waist circumference and IGF-1 receptor tumor status

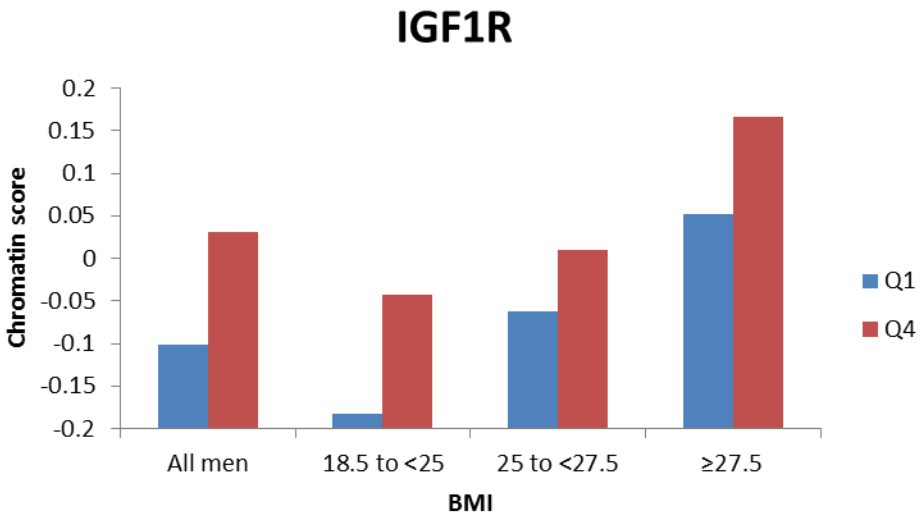


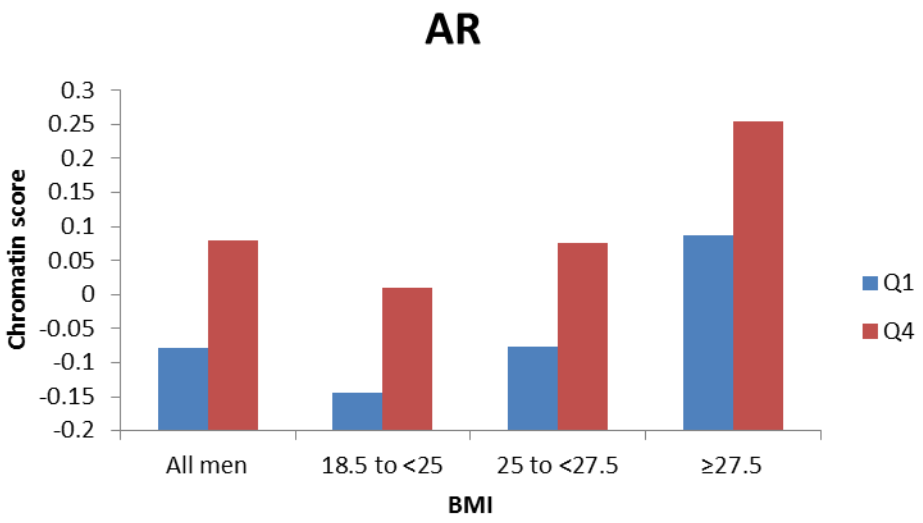
Aim 3: Perform a discovery analysis on the association between obesity and lethal prostate cancer using gene expression data.

We analyzed whole genome mRNA expression profiling data from tumor and adjacent normal tissue to find previously undiscovered biological processes involved in the obesity-lethal cancer relationship. The results of this study are included in the manuscript "Gene expression profiling identifies chromatin regulation as a molecular link between obesity and lethal prostate cancer" which has been submitted most recently to European Urology (see Appendix 4). Briefly, Gene Set Enrichment Analysis identified fifteen gene sets upregulated in the tumor tissue of overweight/obese prostate cancer patients (BMI ≥ 27.5 kg/m^2; N=84) compared to healthy weight patients (BMI 18.5 to < 25 kg/m^2; N=192), five of which were related to chromatin modification and remodeling. Strikingly, these features were not found when comparing normal prostatic adjacent tissues of obese patients with healthy weight patients, suggesting that BMI might exert epigenetic modification only in cancer settings. Importantly, patients with high tumor expression of chromatin-related genes had worse clinical characteristics; 40.6% of men with high expression had Gleason grade >7 cancer compared to 16.8 with low expression (p-value = 3.21 x 10^{-4}). In addition, men with higher tumor expression of chromatin-related genes had a significantly increased risk of metastases or death from prostate cancer, independent of age and year at diagnosis, with an odds ratio of 6.78 (95% confidence interval = 3.42 to 14.16) for lethal outcome comparing extreme quartiles of expression. Of note, a number of the genes identified in this analysis were histone modifying enzymes, including acetyltransferases (KAT2A), deacetylases (HDAC 2,3,8 and SIRT1), methyltransferases (CARM1 and SUV39H2), and methylases (KDM4A). While these results warrant further study, they suggest that obesity may promote the metastatic potential of prostate cancer by influencing its histone profile. To explore this hypothesis further, we tested the relationship between the chromatin gene score and obesity-related tissue biomarkers. We identified several biomarkers associated with the score including the adiponectin receptor (ADIPOR2), IGF1R, and androgen receptor (AR). None of these associations were modified by BMI. (Figure 2, unpublished data)

Figure 2. Mean chromatin gene expression score among the highest quartile (Q4) and lowest quartile (Q1) of biomarker expression for all men and according to BMI status







To explore further the BMI GSEA results described above, we tested the association between each of the gene sets identified and lethal prostate cancer using logistic regression analysis. Table 3 provides the odds ratios (ORs) for each BMI-enriched gene set score and lethal outcome. We found significant associations for gene sets involved in chromatin regulation, cellular disassembly, RNA processing, and ribonucleotide metabolic process. In a mediation analysis, we found that these gene sets explained 36%, 28%, 19% and 3% of the association between BMI and lethal prostate respectively.

Table 3. Odds ratios (95% CIs) for the association between BMI-enriched gene sets and lethal prostate cancer

Gene set score	OR (95% CI)	P-value
Chromatin regulation	1.22 (1.14, 1.31)	4.13E-08
Cellular disassembly	1.19 (1.10, 1.29)	3.66E-05
RNA processing	1.12 (1.05, 1.21)	0.001
Ribonucleotide metabolic process	1.12 (1.06, 1.18)	1.30E-04
Golgi vesicle transport	1.04 (0.99, 1.09)	0.103
Tube development	0.99 (0.93, 1.05)	0.733

What opportunities for training and professional development has the project provided?
This award has provided many opportunities to enhance the professional development of the PI. The coursework and research activities accomplished over the past two years have strengthened Dr. Ebot's research skills related to the incorporation of tissue-level biomarker data into epidemiologic studies of prostate cancer progression. Furthermore, the PI has gained experience in preparing grants and manuscripts and has enhanced her communication skills through oral and poster presentations at numerous meetings and conferences. In addition, she has increased her professional network by forming new partnerships with basic science colleagues at the Dana-Farber Cancer Institute which has led to exciting new opportunities to follow up on research findings from this study.

How were the results disseminated to communities of interest?
Results of the gene expression study were highlighted in a research news article on the Prostate Cancer Foundation website (pcf.org) on June 2, 2015.

What you plan to do during the next reporting period to accomplish the goals?
Nothing to report.

IMPACT
What was the impact on the development of the principal discipline(s) of the project?
Results from this study improved our understanding of the risk factors that promote prostate cancer progression and of the underlying biology that gives rise to more aggressive tumors. Specifically, in Aim 3 we identified several BMI-enriched gene signatures associated with poor prognosis. Future studies need to be done to validate these results; however, if confirmed, these findings have the potential to influence the clinical course of men diagnosed with prostate cancer through the identification of biomarkers for high risk disease. Furthermore, the findings from this award have paved the way for additional studies to test how obesity influences histone modifications in prostate cancer. Epigenetic inhibitors that target HDACs have been tested in clinical trials and approved by the US Food and Drug Administration for use in treating specific cancers. Thus, understanding the specific role of obesity-related epigenetic events in prostate cancer progression could lead to new therapeutic targets to prevent or treat prostate cancer in both obese and non-obese men.

What was the impact on other disciplines?
Nothing to report.

What was the impact on technology transfer?
Nothing to report.

What was the impact on society beyond science and technology?
Nothing to report.

CHANGES/PROBLEMS
Changes in approach and reasons for change
Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them
Nothing to report.

Changes that had a significant impact on expenditures
Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to report.

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use or care of vertebrate animals.
Nothing to report.

Significant changes in use of biohazards and/or select agents
Nothing to report.

PRODUCTS
Publications, conference papers, and presentations

Journal publications
Ebot EM, Gerke T, Labbé DP, Sinnott JA, Zadra G, Rider JR, Tyekucheva S, Wilson KM, Kelly RS, Shui IM, Loda M, Kantoff PW, Finn S, Vander Heiden MG, Brown M, Giovannucci EL, Mucci LA. Gene expression profiling identifies chromatin regulation as a molecular link between obesity and lethal prostate cancer. (submitted – see Appendix 4)

Labbé DP, Zadra G, **Ebot EM**, Mucci LA, Kantoff PW, Loda M, Brown M. Role of diet in prostate cancer: The epigenetic link. Oncogene. 2015 Sep 3;34(36):4683-91. (see Appendix 5)

Books or other non-periodical, one-time publications
Nothing to report.

Other publications, conference papers, and presentations
Identifying obesity-linked gene expression alterations in prostate cancer, Tissue Working Group Meeting, Channing Division of Network Medicine, Brigham and Women's Hospital, Boston, MA, March 29, 2016 (oral presentation – see Appendix 3)

Gene expression profiling identifies chromatin regulation as a molecular link between obesity and lethal prostate cancer, Patho-epidemiology Mini Retreat, HSPH, Boston, MA, February 22, 2016 (oral presentation – see Appendix 2)

Using gene expression profiles of prostate cancer tissue to investigate the relationship between obesity and lethal prostate cancer, ToPCaP Retreat, Dublin, Ireland, September 10, 2015 (oral presentation – see Appendix 1)

Using gene expression profiles of prostate cancer tissue to investigate the relationship between obesity and lethal prostate cancer, Meeting on Lipid Metabolism and Metabolic Alterations in Prostate Cancer, Dana-Farber Cancer Institute, Boston, MA, July 31, 2015 (oral presentation – see annual progress report Appendix 4)

Identifying obesity-linked gene expression changes in prostate cancer, AACR Metabolism and Cancer Conference, Bellevue, WA, June 7-10, 2015 (poster presentation – see annual progress report Appendix 6)

Identifying obesity-linked gene expression changes in prostate cancer, AACR Annual Meeting 2015, Philadelphia, PA, April 18-22, 2015 (oral presentation – see annual progress report Appendix 5)

Obesity and chromatin remodeling – is there an epigenetic link between diet and prostate cancer, Eighth Annual Prostate Cancer SPORE Retreat, Fort Lauderdale, FL, March 15-17, 2015 (oral presentation – see annual progress report Appendix 1)

Identifying obesity-linked gene expression changes in prostate cancer, Program in Genetic Epidemiology and Statistical Genetics Seminar Series, Boston, MA, February 13, 2015 (oral presentation – see annual progress report Appendix 3)

Identifying obesity-linked gene expression changes in prostate cancer, DF/HCC Celebration of Junior Investigators in Cancer Science, Boston, MA, September 24, 2014 (oral presentation – see annual progress report Appendix 2)

Website(s) or other Internet site(s)
Nothing to report.

Technologies or techniques
Nothing to report.

Inventions, patent applications, and/or licenses
Nothing to report.

Other Products
Nothing to report.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS
What individuals have worked on the project?
Ericka Ebot (Noonan) – no change

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Past support:
Concluded
1) Molecular Epidemiology Investigation of Obesity and Lethal Prostate Cancer (DoD, PI Ericka Noonan)
2) Statistical methods for tumor expression data from archival tissues in clinical and epidemiologic research (NIH, PI Svitlana Tyekucheva)

Current support (as of 2/1/17):
1) Circadian Disruption and Risk of Prostate Cancer in a Multiethnic Cohort (NIH, PI Lorelei Mucci)
2) Obesity, histone modifications and lethal prostate cancer (HSPH, PI Lorelei Mucci)
3) Prostate Cancer Research Support Award (DFCI, PI Ericka Ebot)
4) Emory, Harvard & Univ. of Washington Prostate Cancer Biomarker Center (NIH/NCI, PI Lorelei Mucci)
5) Developing a PTEN-ERG Signature to Improve Molecular Risk Stratification in Prostate Cancer (DoD, PI Luigi Marchionni)

What other organizations were involved as partners?
Nothing to report.

SPECIAL REPORTING REQUIREMENTS
Nothing to report.

APPENDICES
1. Presentation: ToPCaP Retreat
2. Presentation: Patho-epidemiology Mini Retreat
3. Presentation: Tissue Working Group Meeting
4. Manuscript: Gene expression profiling identifies chromatin regulation as a molecular link between obesity and lethal prostate cancer
5. Manuscript: Role of diet in prostate cancer: The epigenetic link

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4. Cao Y, Ma J. Body mass index, prostate cancer-specific mortality, and biochemical recurrence: a systematic review and meta-analysis. *Cancer Prev Res (Phila).* Apr 2011;4(4):486-501.
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Using gene expression profiles of prostate cancer tissue to investigate the relationship between obesity and lethal prostate cancer

Ericka Ebot

Postdoctoral Fellow, Epidemiology

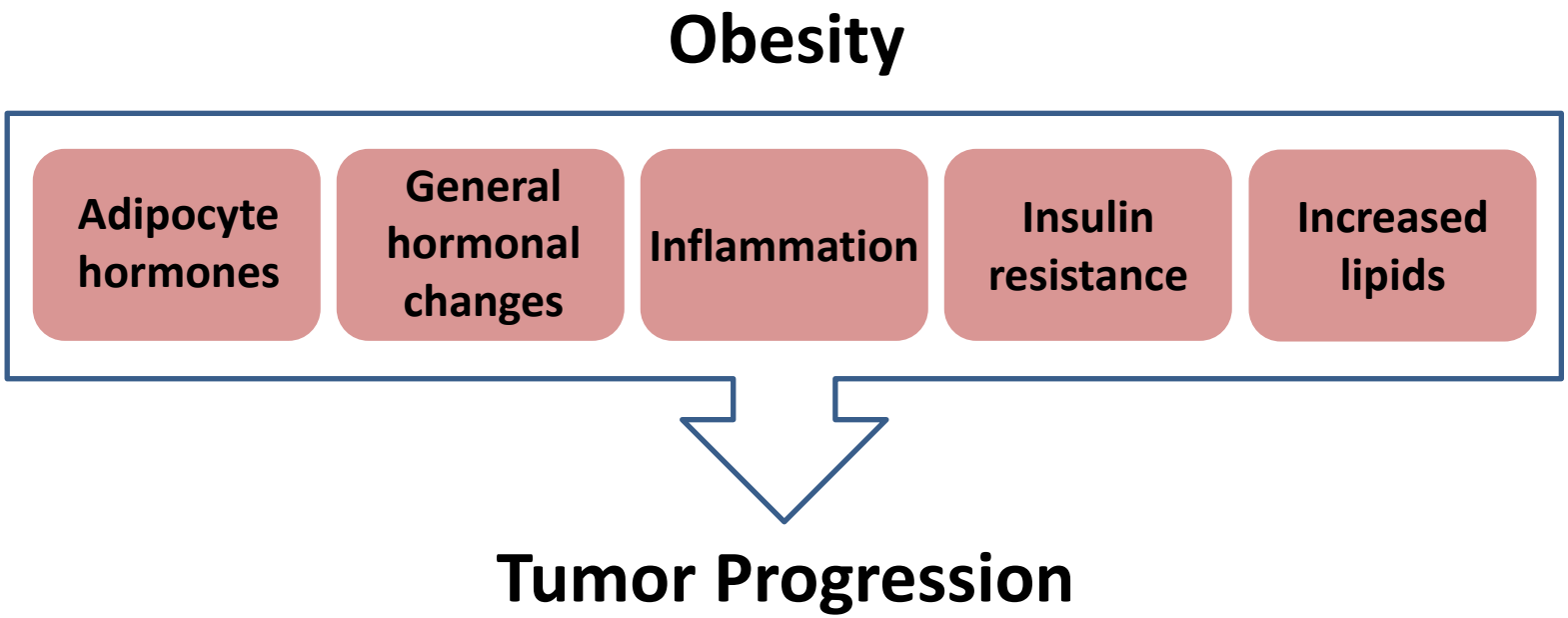
Harvard T.H. Chan School of Public Health

ToPCaP Retreat

September 10, 2015

Obesity and prostate cancer

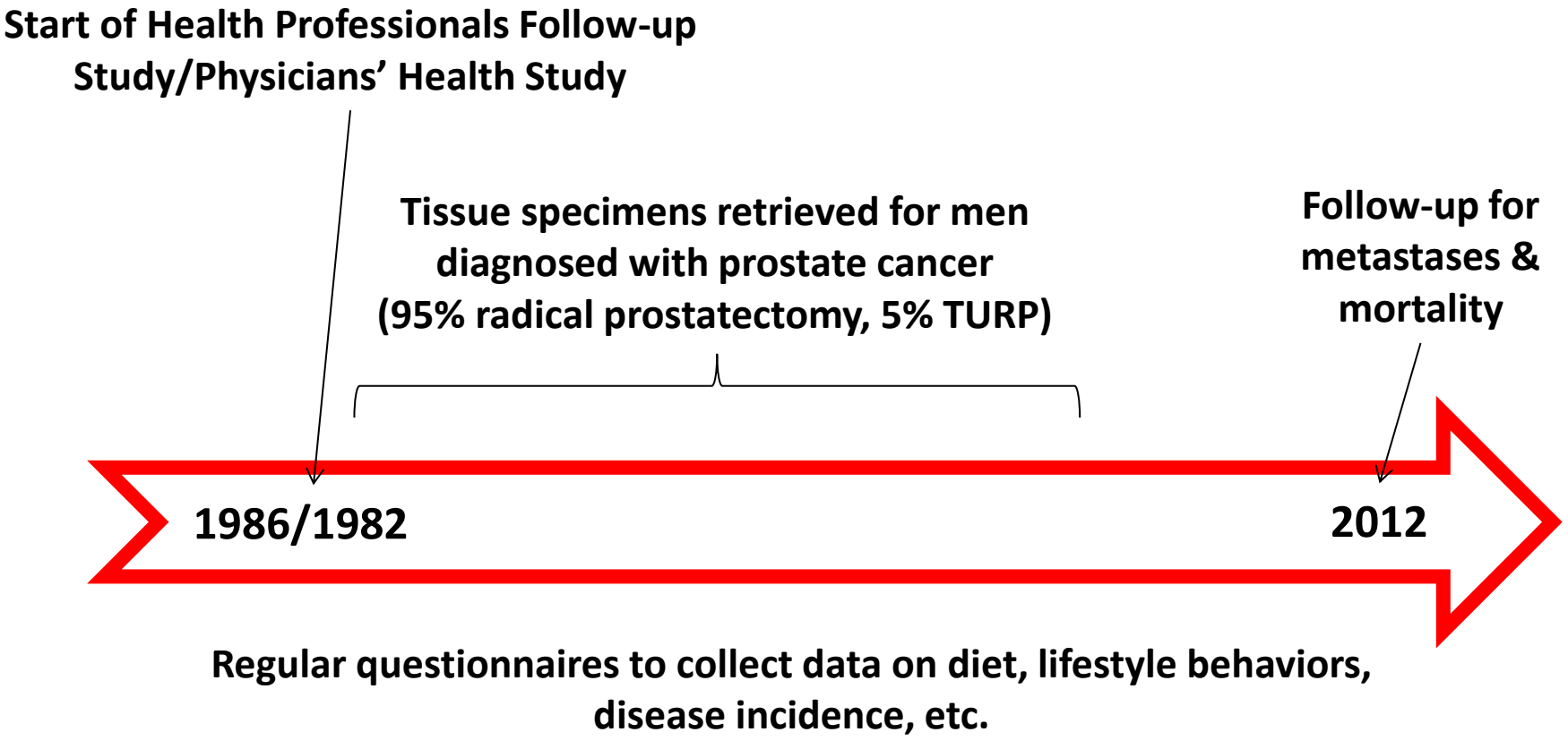
- Obese men are at higher risk of developing advanced stage prostate cancer and have higher rates of cancer-specific mortality after diagnosis



Aims

- Examine prostate-specific alterations associated with obesity using whole transcriptome gene expression profiles of tumor tissue
- Explore whether such alterations underlie the link between obesity and lethal disease

Harvard Prostate Tumor Tissue Cohort



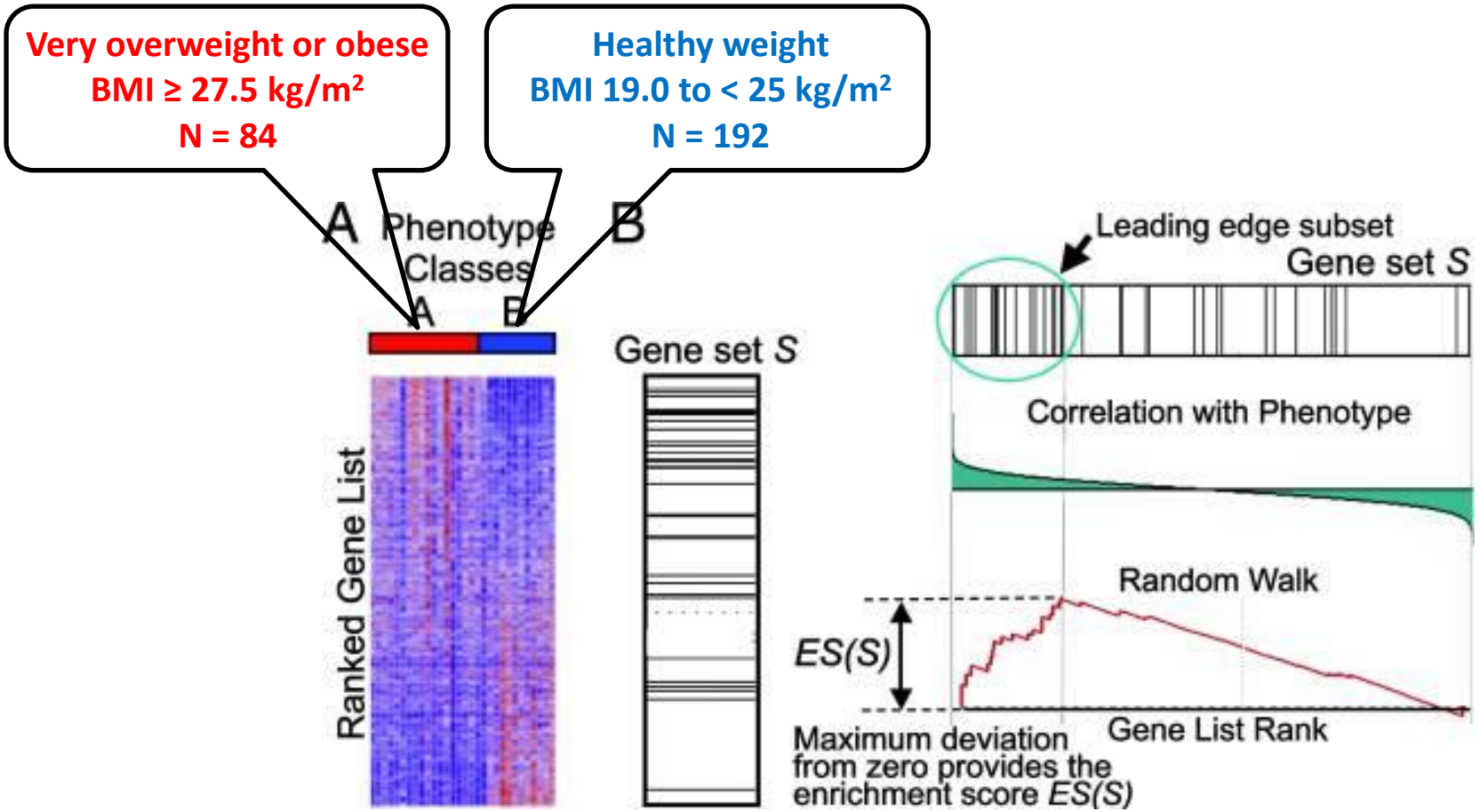
Methods

- **Study population:** 402 prostate cancer cases from the Harvard Prostate Tumor Tissue Cohort diagnosed between 1982 and 2005
  - 113 lethal (metastatic disease or prostate cancer-specific death)
  - 289 indolent (survived 8 years without lethal event)
- **Obesity measures:** Self-reported body mass index (BMI) was taken from closest questionnaire prior to diagnosis (average = 1.3 years)
- **Clinical data:** Clinical information was obtained from medical record review; Standardized histopathologic review of Gleason grade was performed for each case
- **Outcome data:** Prostate cancer cases were followed through questionnaires for details of clinical course; Deaths were ascertained by searches of the National Death Index
- **Biomarker assessment:** Whole transcriptome gene expression profiles of tumor tissue assayed using the Affymetrix GeneChip Human Gene 1.0 ST Array

Clinical characteristics of prostate cancer cases in study (N=402)

Age at diagnosis, years, mean	65.7
Year of diagnosis, %	
before 1990 (pre-PSA era)	11
1990-1993 (peri-PSA era)	28
after 1993 (PSA era)	61
PSA at diagnosis, ng/ml, median	7.3
Pathologic TNM stage, %	
T2 N0 M0	59
T3 N0 M0	35
T4/N1/M1	5
Gleason grade, %	
2-6	14
3+4	34
4+3	25
8-10	26
Tissue type, %	
Radical prostatectomy	92
TURP	9

Gene Set Enrichment Analysis

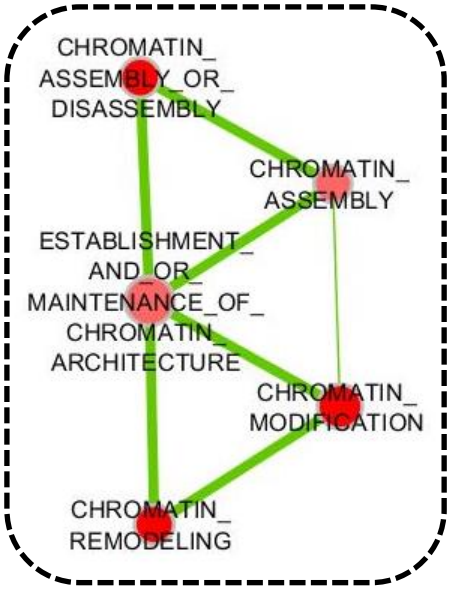


589 Gene Ontology Biological Process gene sets (Molecular Signatures Database, Broad Institute)

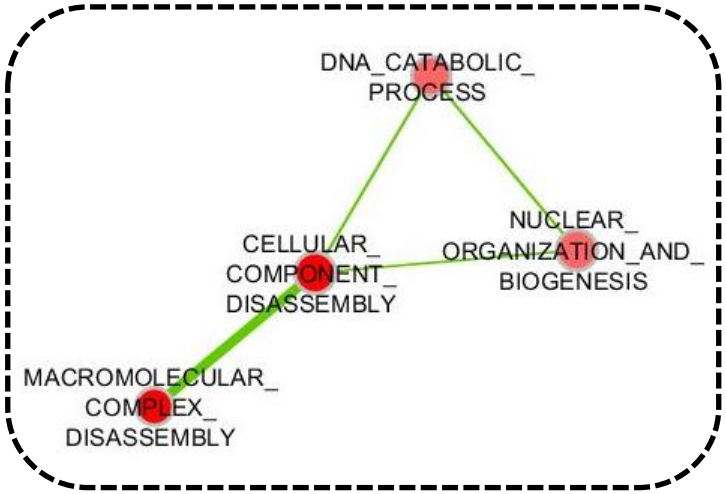
Subramanian et al. PNAS 2005

Top Gene Ontology biological process gene sets

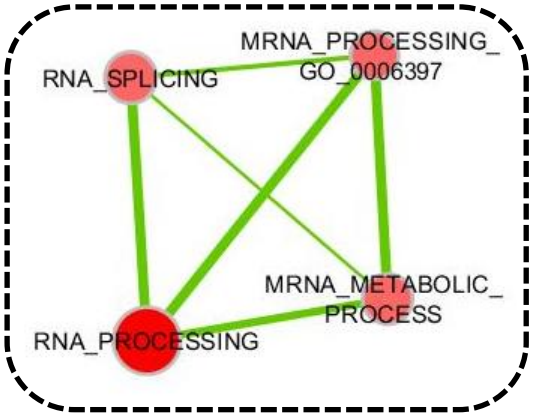
Chromatin regulation (N = 74)



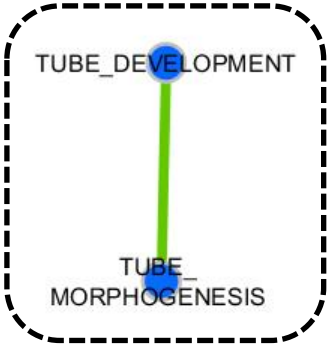
Cellular disassembly (N = 57)



RNA processing (N = 177)



Tube development (N = 17)



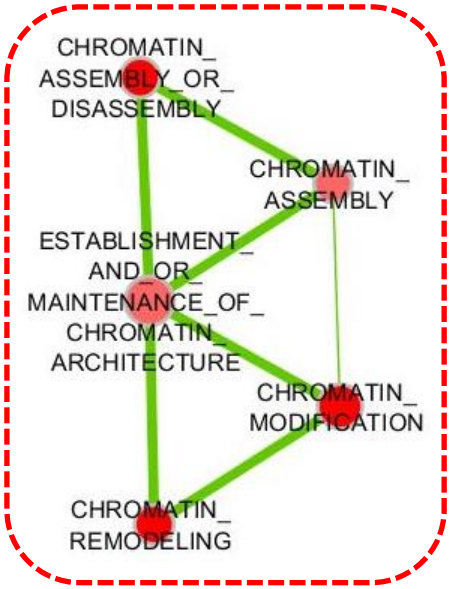
(N = 16)

(N = 48)

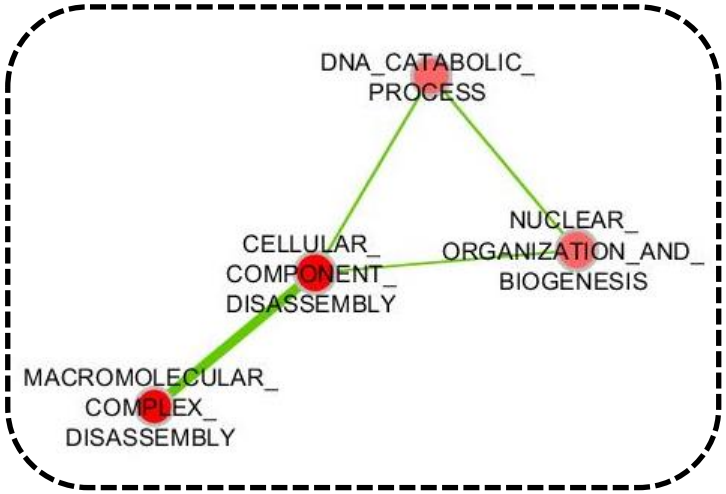
FDR q-value < 0.25

Top Gene Ontology biological process gene sets

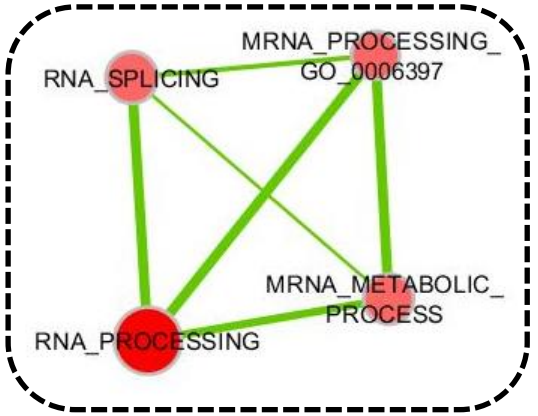
Chromatin regulation (N = 74)



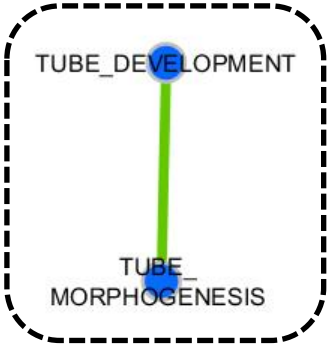
Cellular disassembly (N = 57)



RNA processing (N = 177)



Tube development (N = 17)



RIBONUCLEOTIDE_ METABOLIC_ PROCESS

(N = 16)

GOLGI_VESICLE_ TRANSPORT

(N = 48)

FDR q-value < 0.25

Gene symbol	Gene name
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CARM1	coactivator-associated arginine methyltransferase 1
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Chromatin-related genes from leading edge subset

histone deacetylase activity
HDAC2, HDAC3, HDAC8, SIRT1

nucleosome remodeling
SWI/SNF: SMARCC2, SMARCA5, ARID1A, PBRM1, ACTL6A

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score computed based on expression of chromatin-related genes

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	# lethal	Odds ratio	P-value
Chromatin score			
quartile 1 (low expression)	15	ref	8.11E-05
quartile 2	23	2.03 (0.88, 4.81)	
quartile 3	25	2.04 (0.89, 4.79)	
quartile 4 (high expression)	50	5.01 (2.31, 11.38)	

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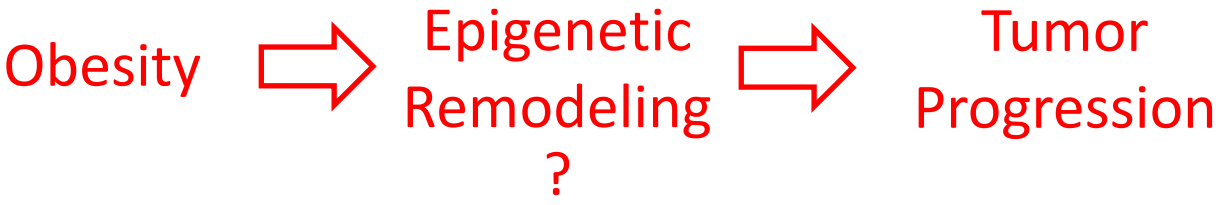

Future directions

- Confirm chromatin remodeling results
  - Validate gene expression findings in independent prostate cancer patient cohort
  - Measure chromatin modifications in prostate tissue
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What can ToPCaP do?

Acknowledgements

Harvard School of Public Health

Lorelei Mucci

Edward Giovannucci

Meir Stampfer

Prostate Cancer Patho-Epi Team

Dana-Farber Cancer Institute

Massimo Loda

Myles Brown

David Labbé

Giorgia Zadra

Funding

DoD Prostate Cancer Research Program

DF/HCC SPORE in Prostate Cancer

Gene expression profiling identifies chromatin regulation as a molecular link between obesity and lethal prostate cancer

Ericka Ebot

Research Associate, Epidemiology

Harvard T.H. Chan School of Public Health

Patho-Epi Mini Retreat

February 22, 2016

Aims

- Examine prostate-specific alterations associated with prediagnosis BMI using whole transcriptome gene expression profiles of tumor tissue

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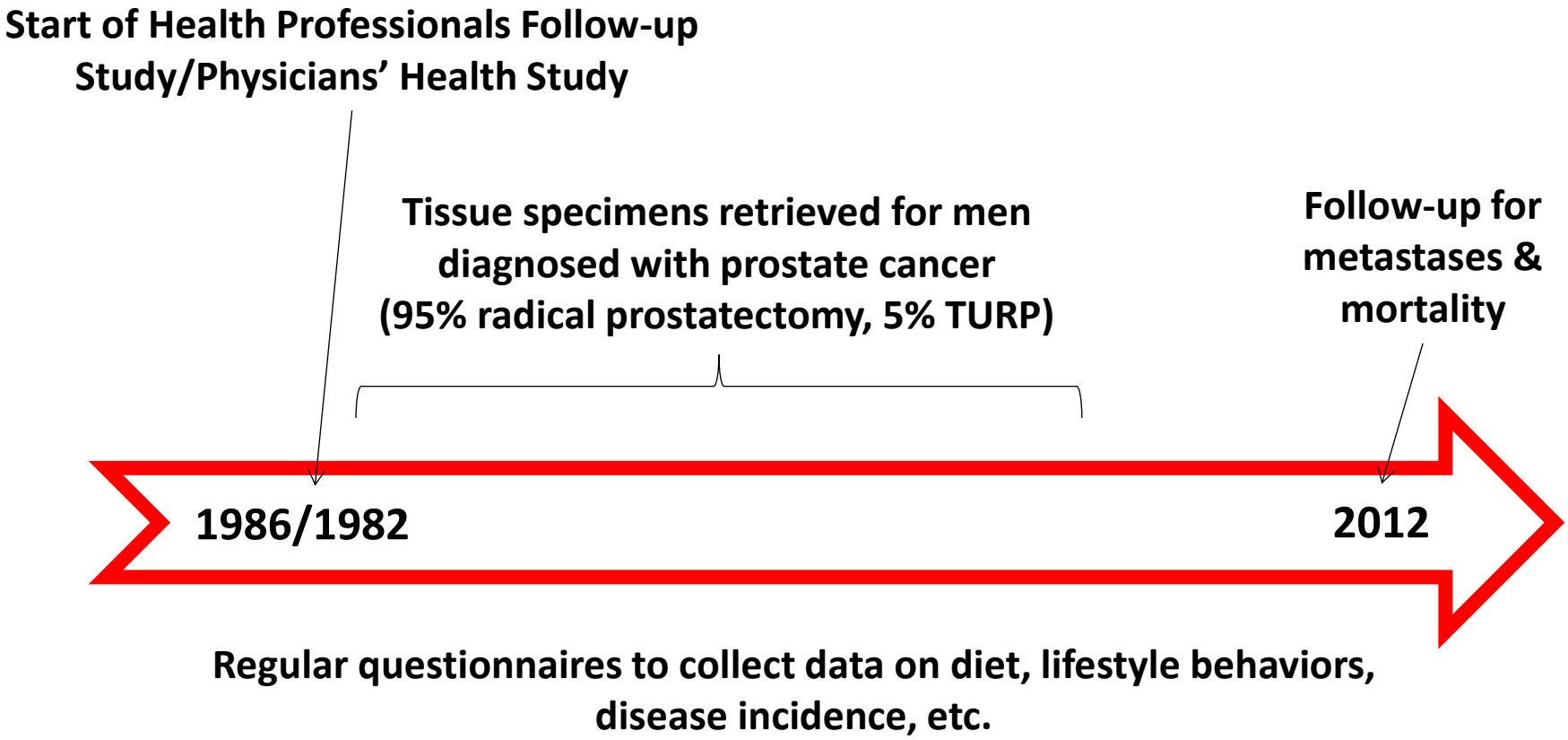


Methods

- **Study population:** 402 prostate cancer cases from the Prostate Tumor Tissue Cohort diagnosed between 1982 and 2005
  - 113 lethal (developed metastatic disease or prostate cancer-specific death)
  - 289 indolent (survived 8 years without lethal event)

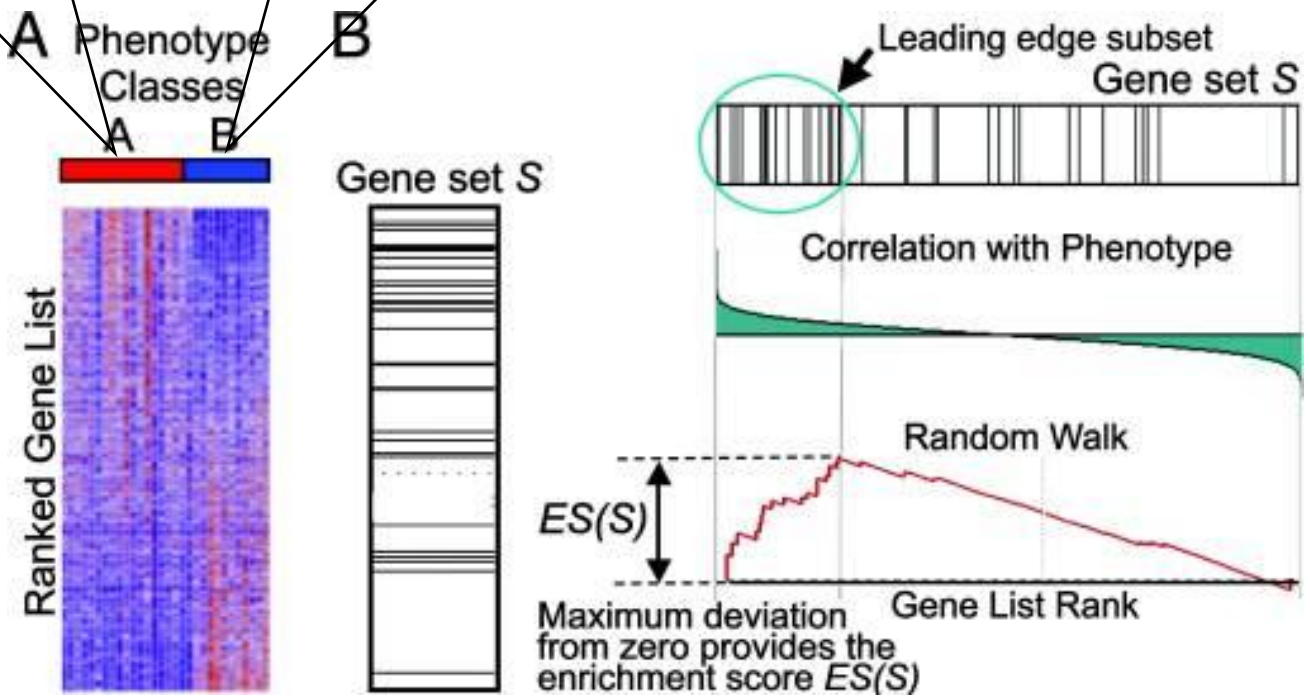
- **Obesity measures:** Self-reported body mass index (BMI) was taken from questionnaires closest to and before diagnosis (average = 1.3 years)

- **Biomarker assessment:** Whole transcriptome gene expression profiles of tumor tissue assayed using the Affymetrix GeneChip Human Gene 1.0 ST Array
  - 20,254 unique gene symbols after mapping transcript cluster IDs to gene names

Gene Set Enrichment Analysis

Very overweight or obese
BMI ≥ 27.5 kg/m²
N = 84

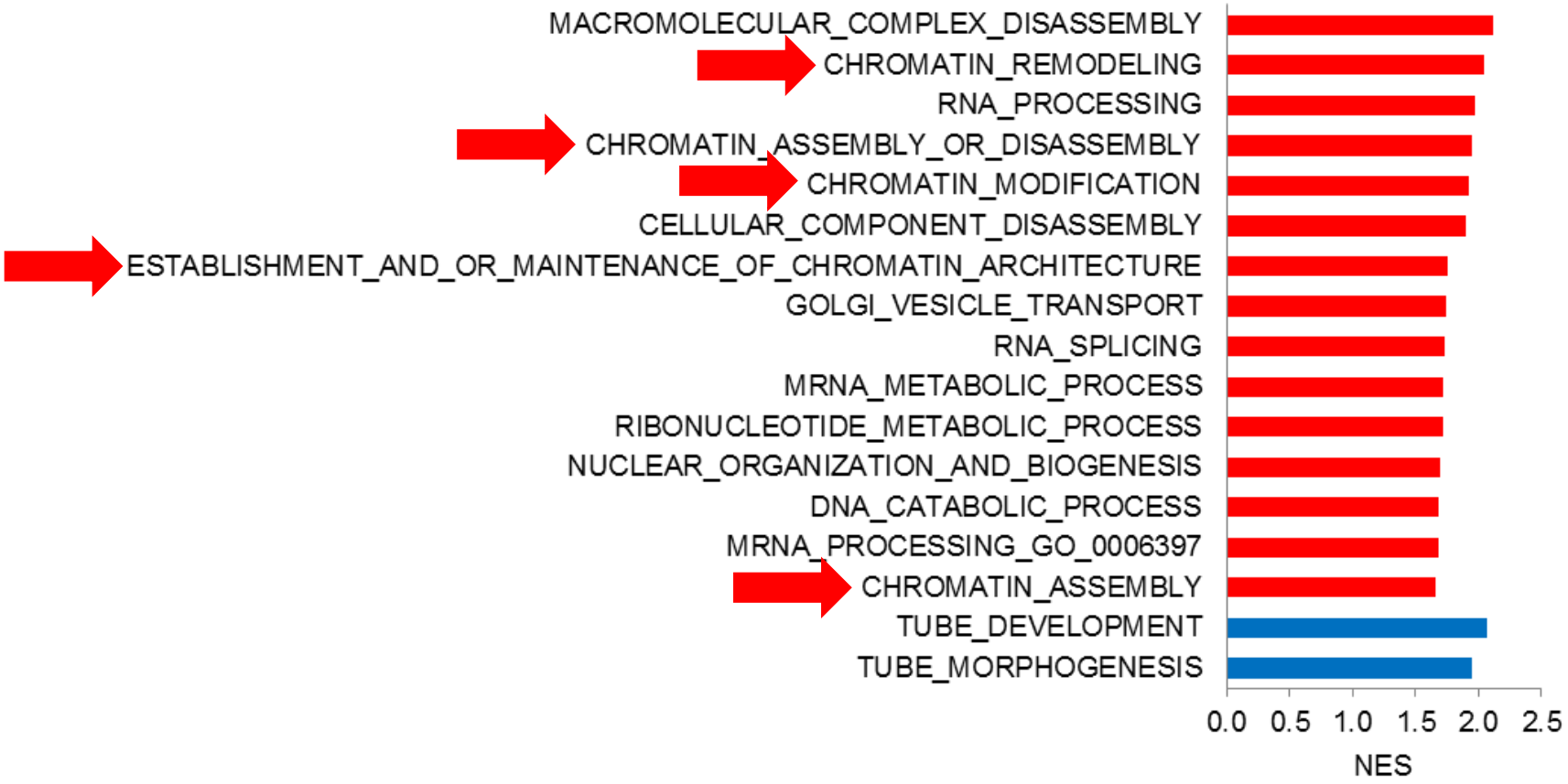
Healthy weight
BMI 19.0 to < 25 kg/m²
N = 192



589 Gene Ontology Biological Process gene sets
(Molecular Signatures Database, Broad Institute)

Subramanian et al. PNAS 2005

Top Gene Ontology biological process gene sets associated with prediagnosis BMI



FDR < 0.25

Increased expression of chromatin genes is associated with lethal outcome

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[1]Logistic regression model adjusted for age and year at diagnosis

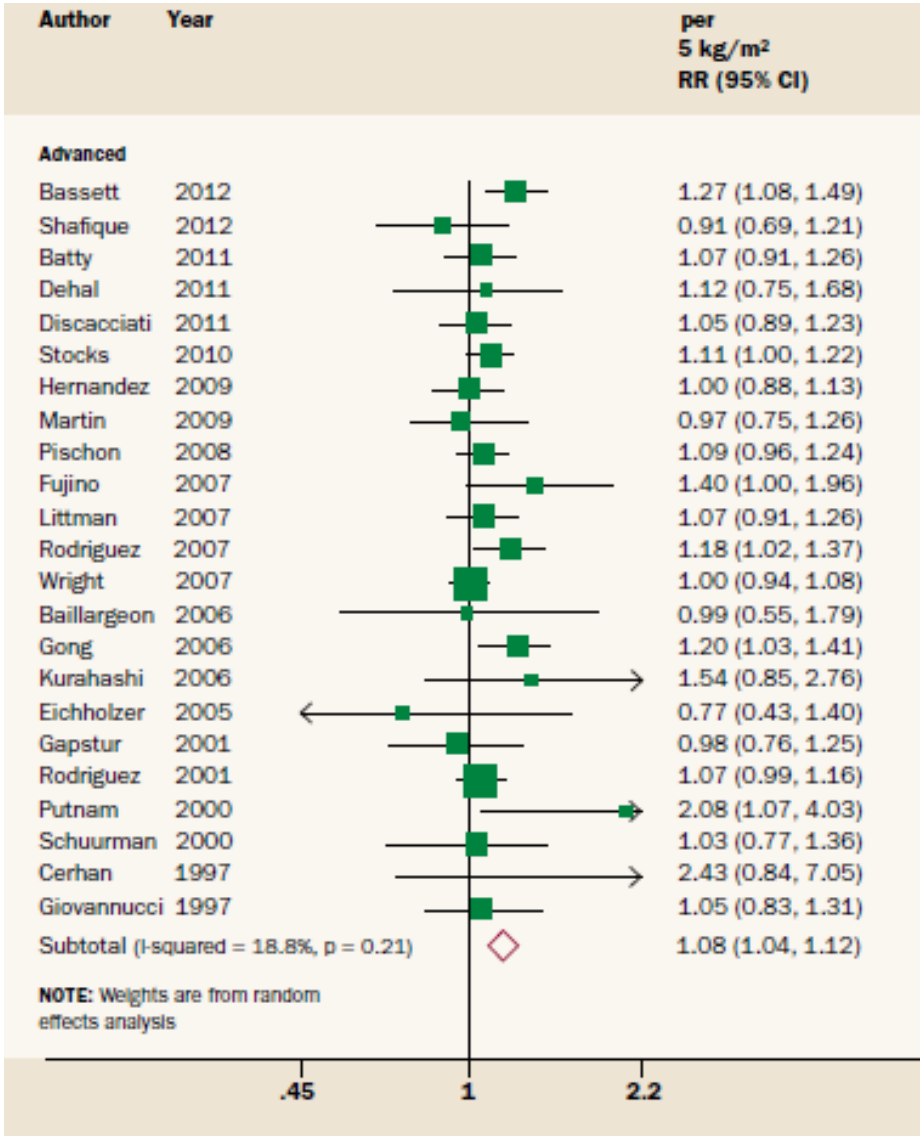
[2]Logistic regression model additionally adjusted for Gleason grade

Identifying obesity-linked gene expression alterations in prostate cancer

Ericka Ebot, PhD, MPH
Research Associate, Epidemiology
Harvard T.H. Chan School of Public Health

Combined Subtyping/Tissue Working Group Meeting
Channing Division of Network Medicine
March 29, 2016

Meta-analysis of BMI and advanced prostate cancer

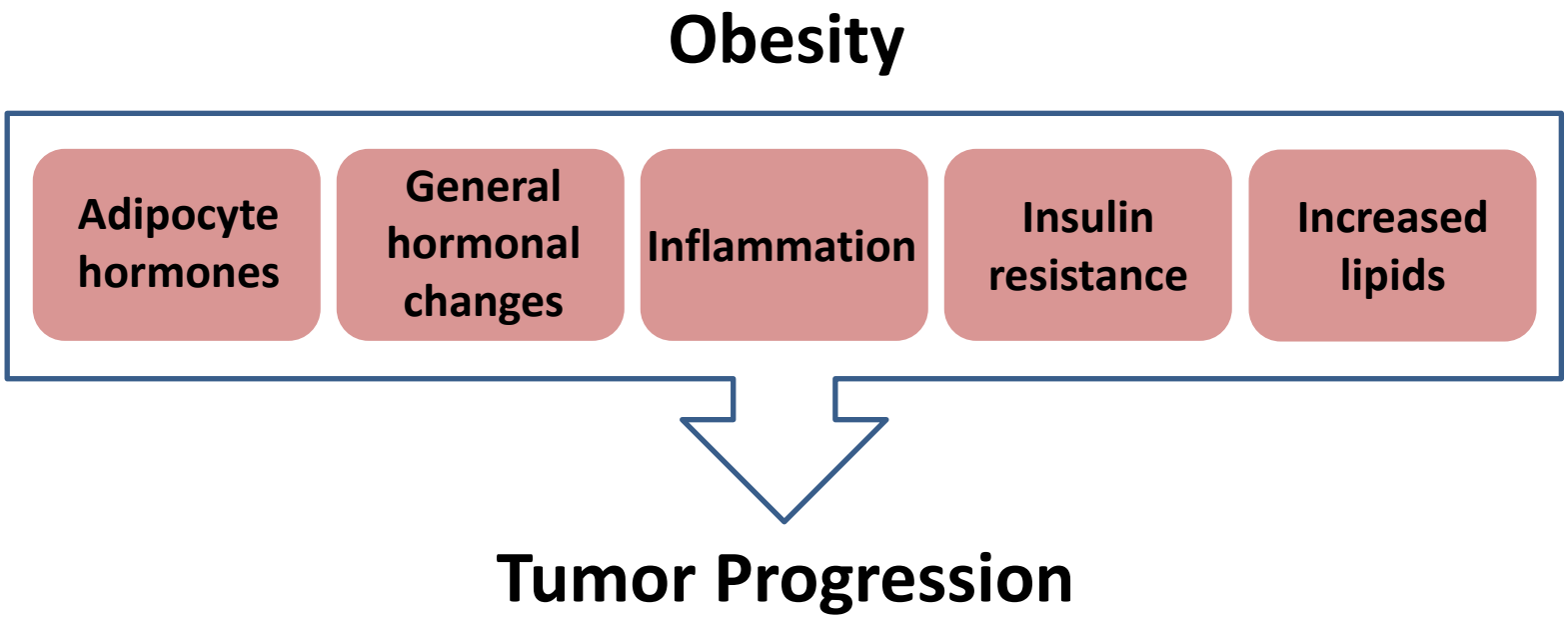


- **8% increased risk of advanced prostate cancer per 5kg/m² increase in BMI**
- **11% increased risk of prostate cancer mortality per 5kg/m² increase in BMI**

WCRF/AICR 2014 Report on Diet, Nutrition, Physical Activity, and Prostate Cancer

Obesity and aggressive prostate cancer

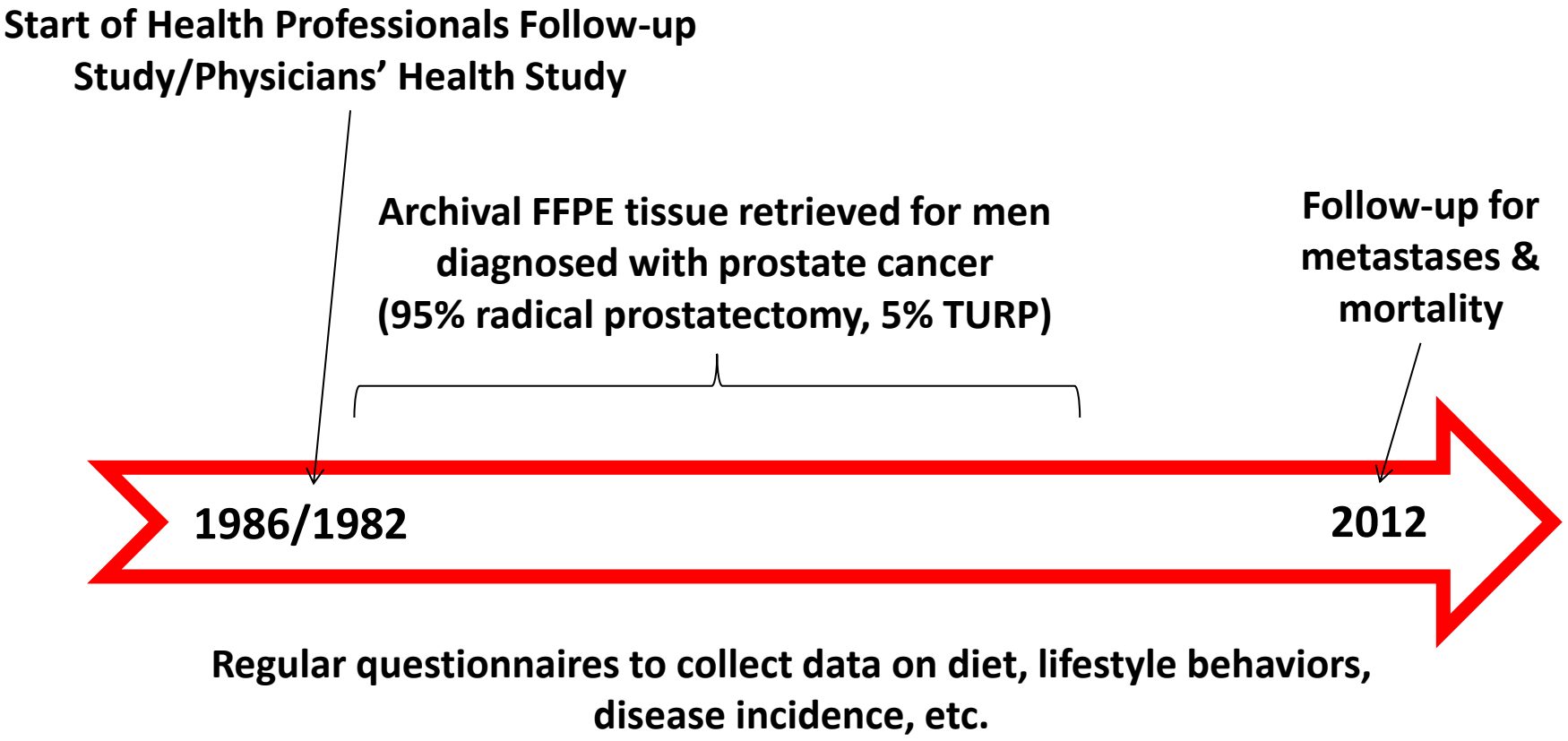
- Overweight and obese men are at higher risk of developing advanced stage prostate cancer and have higher rates of cancer-specific mortality after diagnosis



Aims

- Examine prostate-specific alterations associated with obesity using whole transcriptome gene expression profiles of tumor tissue
- Explore whether such alterations underlie the link between obesity and lethal disease

Prostate Tumor Tissue Cohort



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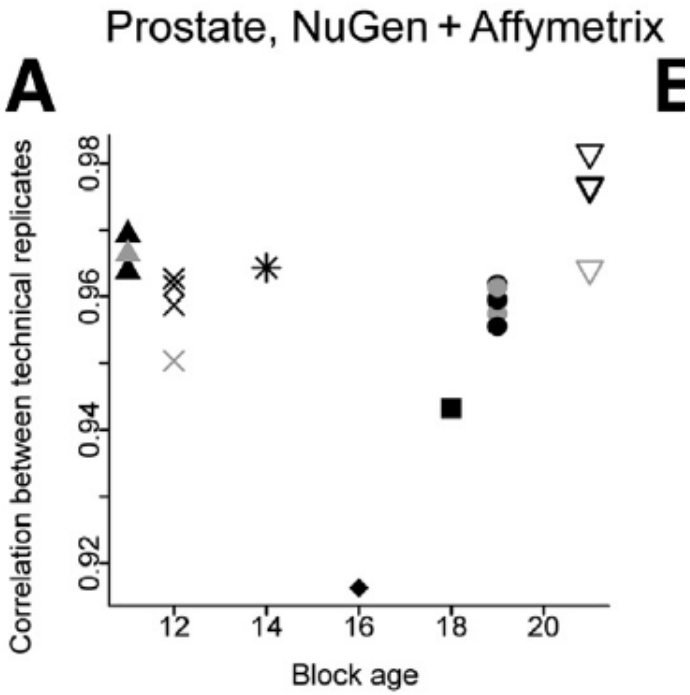
  - 289 indolent (survived 8 years without lethal event)
- **Obesity measures:** Self-reported body mass index (BMI) taken from questionnaires closest to and before diagnosis (average = 1.3 years)
- **mRNA expression profiling:**
  - Whole-transcriptome amplification and array hybridization performed using the WT-Ovation FFPE System V2 (NuGen) and GeneChip Human Gene 1.0 ST Array (Affymetrix)
  - Data normalized using modified Robust Multichip Average method (regressed out technical variables including mRNA concentration, block age, batch (96-well plate), background signal)
  - 20,254 unique gene symbols after mapping transcript cluster IDs to gene names

NuGen + Affymetrix profiling method demonstrated high correlation across technical replicates (independent of FFPE block age)

Table 1 Ranges of the Percentages of the Probes with the Signal above the Background, Correlation of the Percentage of Present Probes with Block Age, and Ranges of Correlations between Technical Replicates for Each Cohort and Gene Expression Platform

Platform	Percent present	Correlation with block age	Correlations between technical replicates
Prostate samples			
NuGen + Affymetrix	0.17—0.58	0.02	0.91—0.98
NanoString	0.40—0.79	−0.11	0.88—0.97
Ovarian samples			
NuGen + Affymetrix	0.40—0.68	0.06	0.94—0.98
NanoString	0.83—0.93	−0.04	0.95—0.99



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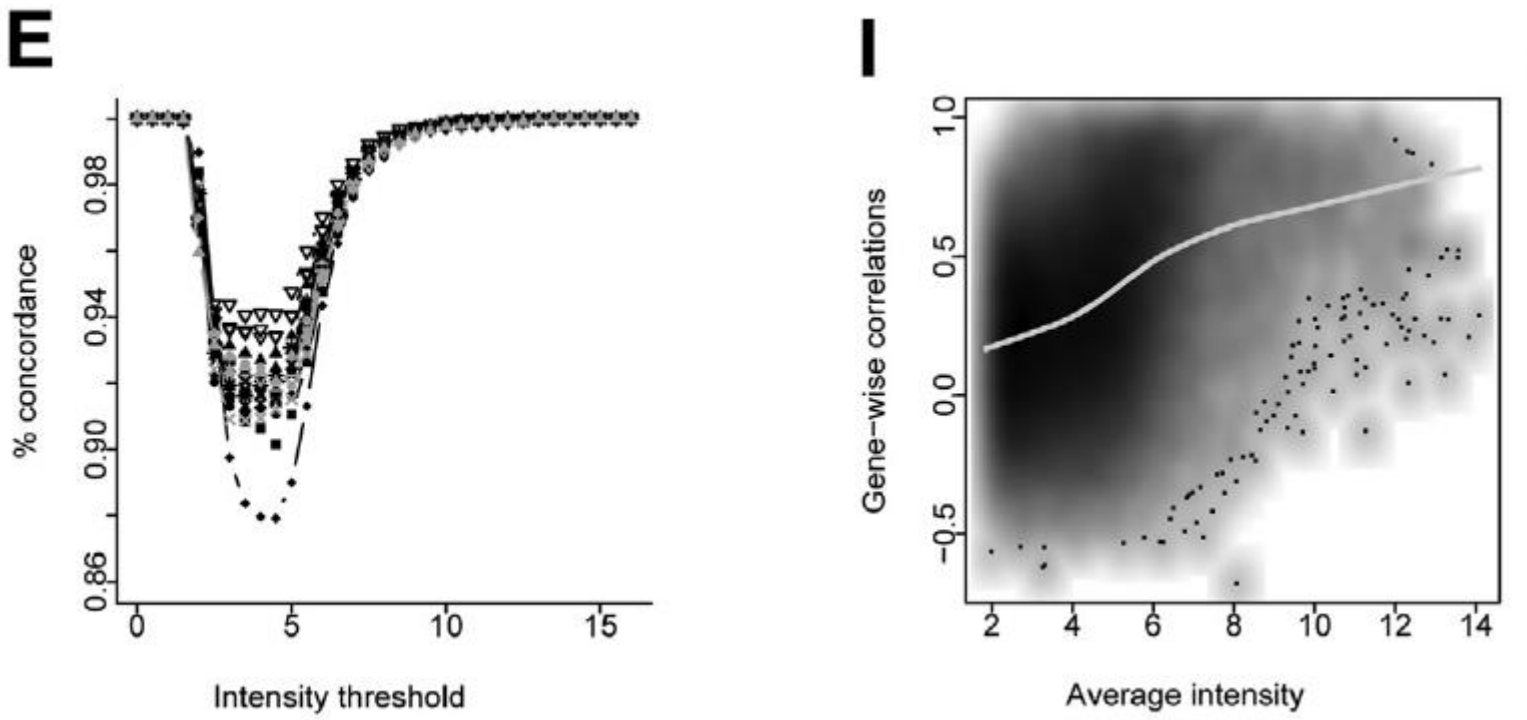

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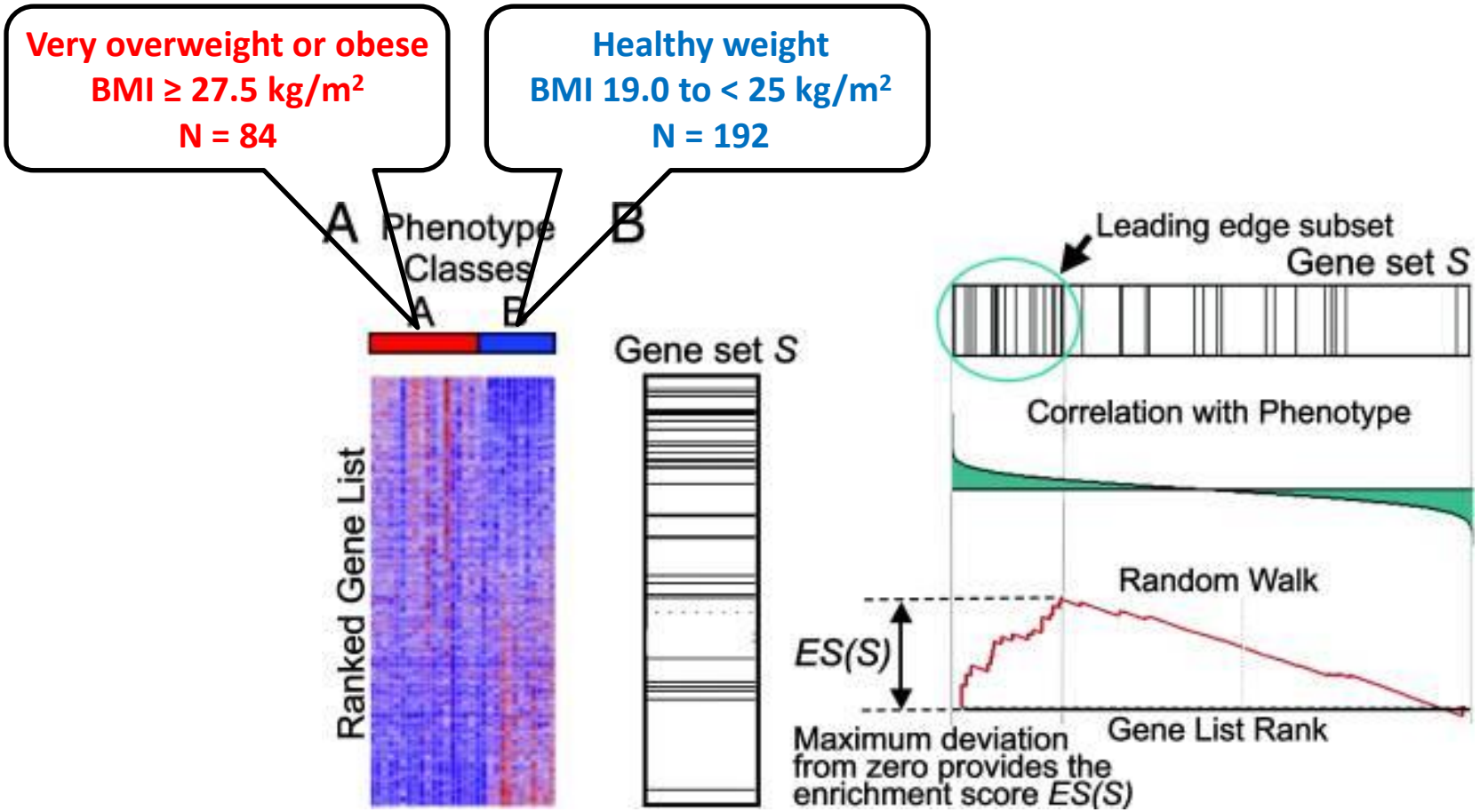
Clinical characteristics of prostate cancer cases in study (N=402)

Age at diagnosis, years, mean	65.7
Year of diagnosis, %	
before 1990 (pre-psa era)	11
1990-1993 (peri-psa era)	28
after 1993 (psa era)	61
PSA at diagnosis, ng/ml, median	7.3
Pathologic TNM stage, %	
T2 N0 M0	59
T3 N0 M0	35
T4/N1/M1	5
Gleason grade, %	
2-6	14
3+4	34
4+3	25
8-10	26

Gene Set Enrichment Analysis



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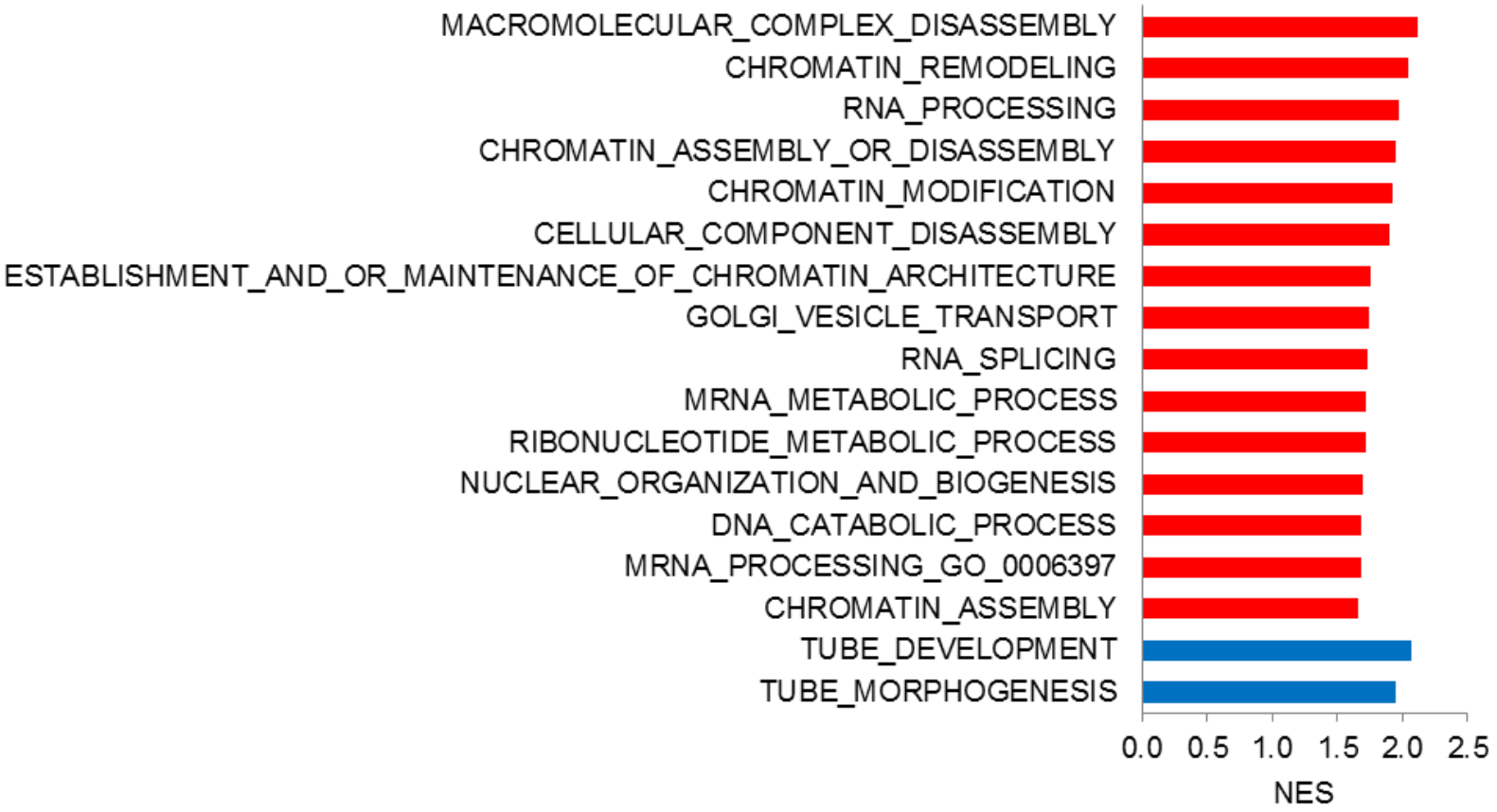

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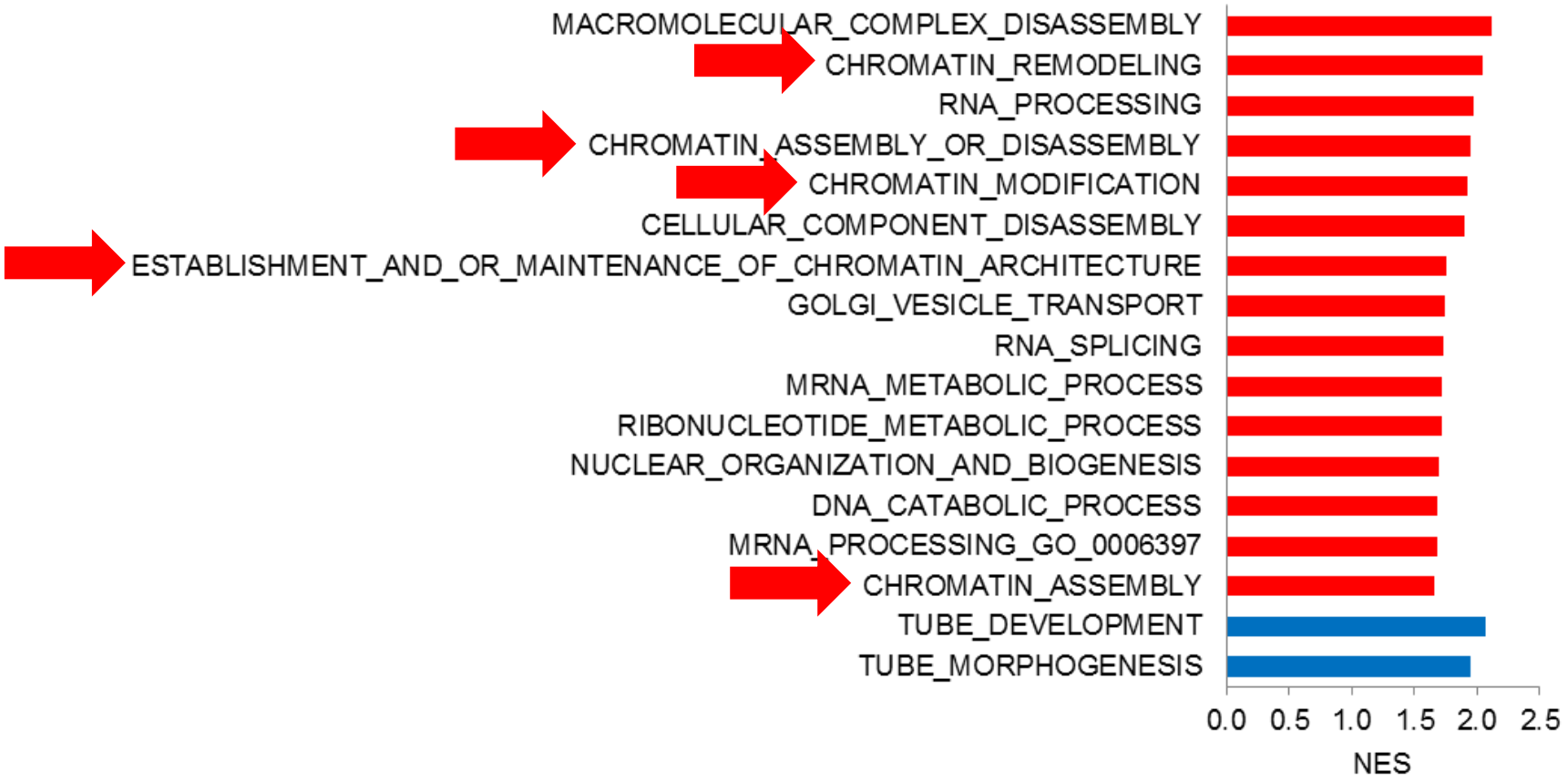

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Enrichment Map of gene sets with FDR < 0.25

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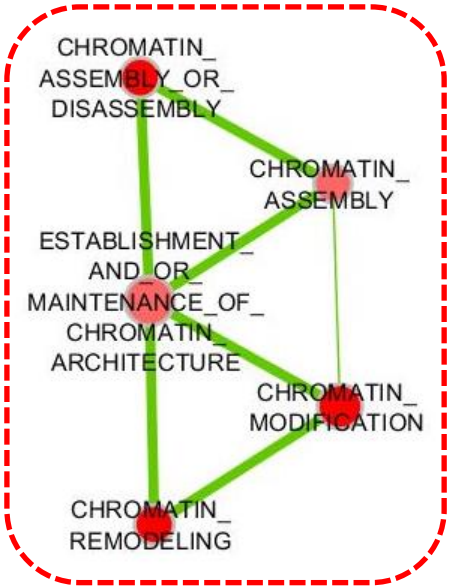

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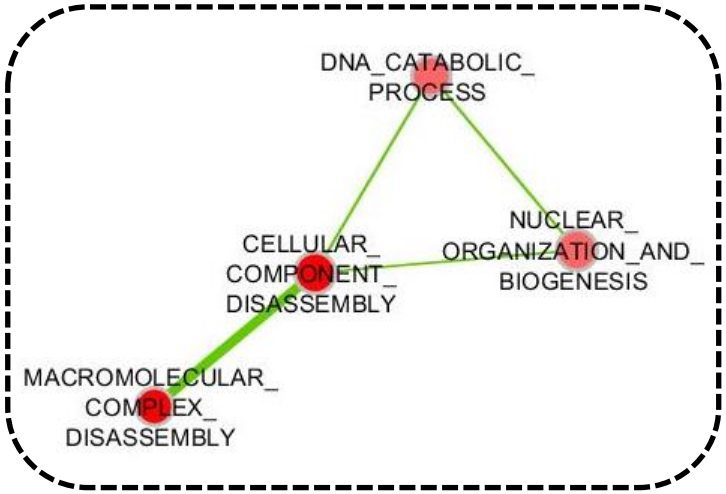

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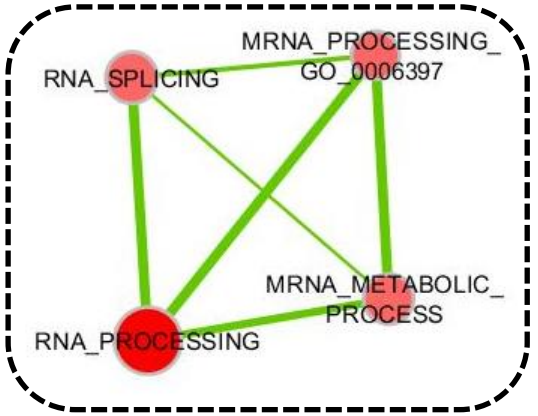

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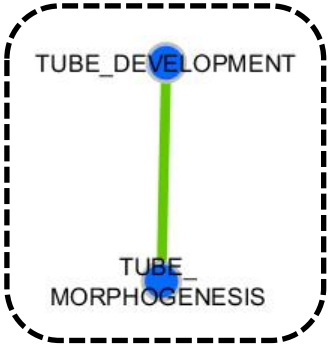

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score computed based on expression of chromatin genes

Increased expression of chromatin genes is associated with worse clinical characteristics

	Chromatin gene score		
	Quartile 1 (low expression)	Quartile 4 (high expression)	
Age at diagnosis, years, mean	66.7	66.5	
Year of diagnosis, %			
before 1990 (pre-PSA era)	11	13	
1990-1993 (peri-PSA era)	29	31	
after 1993 (PSA era)	60	56	
PSA at diagnosis, ng/ml, median	6.7	8.1	
Pathologic TNM stage, %			**P-value**
T2 N0 M0	61	49	**0.11**
T3 N0 M0	34	45	
T4/N1/M1	4	6	
Gleason grade, %			
2-6	20	9	
3+4	39	18	**P-value**
4+3	25	33	**3.2×10^{-4}**
8-10	17	41	

Increased expression of chromatin genes is associated with lethal outcome

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Chromatin gene score					
Quartile 1 (low expression)	15	ref	1.03E-07	ref	8.11E-05
Quartile 2	23	2.13 (1.02, 4.57)		2.03 (0.88, 4.81)	
Quartile 3	25	2.25 (1.08, 4.82)		2.04 (0.89, 4.79)	
Quartile 4 (high expression)	50	6.78 (3.42, 14.16)		5.01 (2.31, 11.38)	

[1]Logistic regression model adjusted for age and year at diagnosis

[2]Logistic regression model additionally adjusted for Gleason grade

Increased expression of chromatin genes is associated with lethal outcome

	# lethal	Odds ratio[1]	P-value	Odds ratio[2]	P-value
Chromatin gene score					
Quartile 1 (low expression)	15	ref	1.03E-07	ref	8.11E-05
Quartile 2	23	2.13 (1.02, 4.57)		2.03 (0.88, 4.81)	
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[1]Logistic regression model adjusted for age and year at diagnosis

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Summary

- Genes involved in chromatin modification and remodeling are enriched in the tumor tissue of overweight/obese prostate cancer patients
  - Tumor-specific
- Expression of chromatin-related genes is associated with worse tumor characteristics (Gleason grade) and poorer survival
- Expression of chromatin-related genes is associated with greater levels of proliferation (Ki-67) and angiogenesis (smaller and less regularly shaped vessels)

Summary

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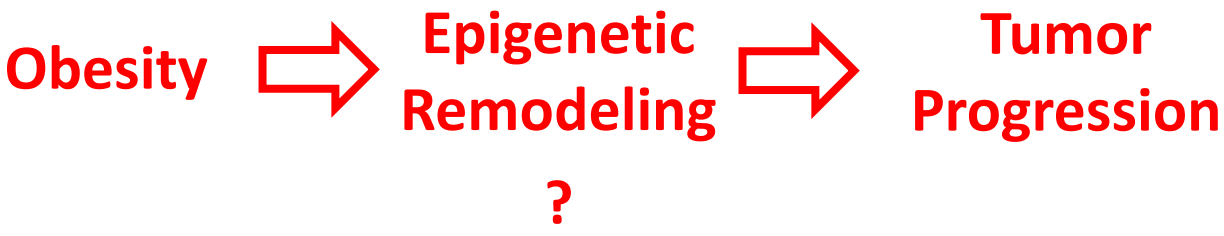
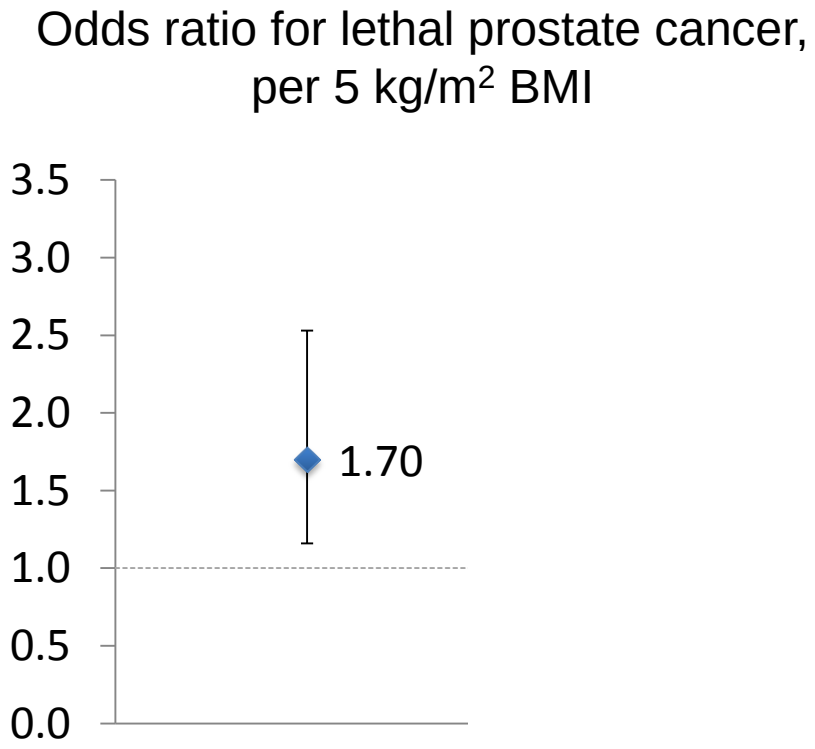


Expression of chromatin genes mediates the relationship between BMI and lethal prostate cancer



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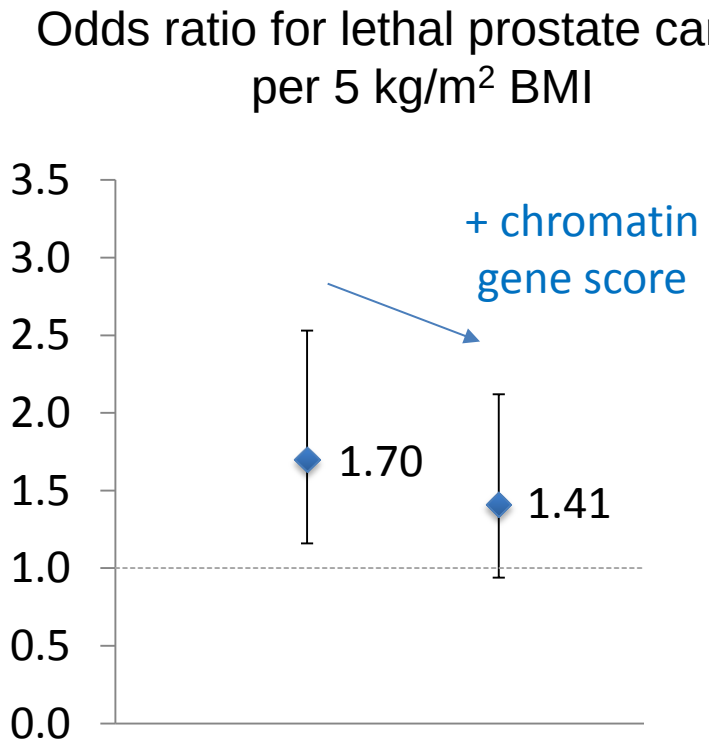

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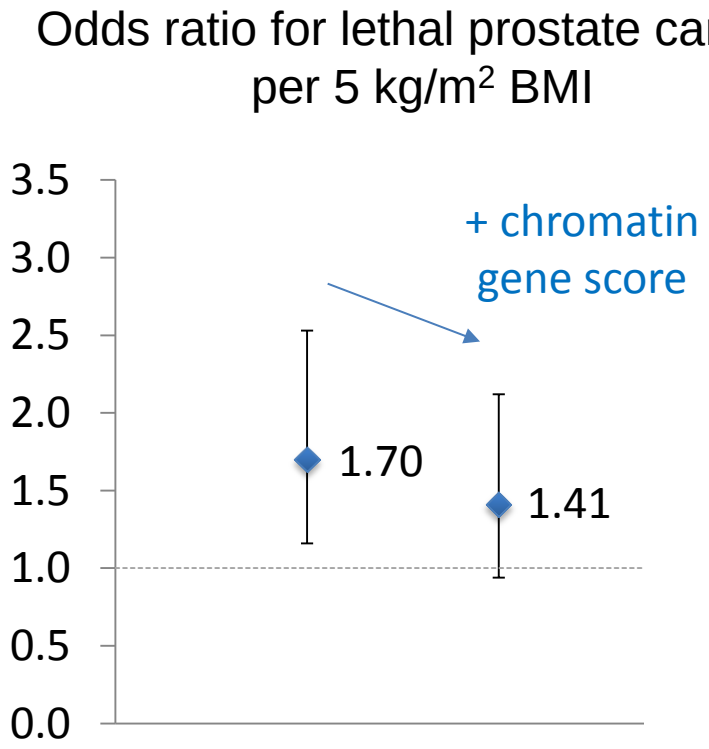

36% of the association between BMI and lethal prostate cancer can be explained by the score

Conclusions

- These results provide support for a causal relationship between obesity and prostate cancer survival and identify a potential target for new treatment or secondary prevention strategies for prostate cancer patients
- Strengths:
  - First human study to look at gene expression alterations in prostate tissue by obesity status and relate such alterations to prostate cancer outcomes
  - Ability to integrate tissue-level biomarker data with exposure and clinical data and long-term follow-up for prostate cancer outcomes
- Limitations:
  - Detection and treatment bias among obese men
  - BMI is an imperfect measure of body fatness

Ongoing work

- Perform global chromatin profiling by quantitative targeted mass spectrometry to identify specific histone post translational modifications in prostate tissue associated with obesity
  - Chromatin findings supported by experimental evidence using a diet-induced obesity mouse model of prostate cancer (David Labbé and Giorgia Zadra, Dana-Farber Cancer Institute)
- Evaluate chromatin gene score in relation to other prostate cancer risk factors

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Gene expression profiling identifies chromatin regulation as a molecular link between obesity and lethal prostate cancer

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Abstract

Background: Obese men are at higher risk of advanced prostate cancer and cancer-specific mortality; however, the biology underlying this association is unclear.

Objective: Examine whether prediagnosis body mass index (BMI) is associated with gene expression profiles in prostate tissue, and whether these profiles explain the link between obesity and lethal prostate cancer.

Design, Setting, and Participants: Gene expression profiling of tumor (N=402) and adjacent normal (N=200) prostate tissue from participants of two cohorts, the Health Professionals Follow-up Study and Physicians' Health Study, diagnosed with prostate cancer from 1982–2005. BMI calculated from questionnaire immediately preceding cancer diagnosis.

Outcome Measurements and Statistical Analysis: Men were followed for lethal disease, defined as metastases or prostate cancer-specific death, through 2011. We identified Gene Ontology biological processes differentially expressed by BMI using Gene Set Enrichment Analysis. Pathway scores were computed by averaging signal intensities of member genes. Odds ratios (ORs) and 95% confidence intervals (CIs) for lethal cancer were estimated using logistic regression.

Results and Limitations: Of 402 men, 48% were healthy weight, 31% were overweight, and 21% were very overweight/obese. Fifteen gene sets were enriched in tumor tissue, but not normal tissue, of very overweight/obese vs. healthy weight men; five of which were related to chromatin modification and remodeling (false discovery rate < 0.25). Patients with high tumor expression of chromatin-related genes had worse clinical characteristics (Gleason grade >7, 41% versus 17%, p-value = $3x10^{-4}$) and

increased risk of lethal disease independent of grade (OR = 5.01, 95% CI = 2.31 to 11.38).

Conclusions: Genes involved in chromatin regulation are upregulated in tumor tissue of overweight/obese prostate cancer patients, and their expression is associated with worse clinical characteristics and outcomes. These findings identify a promising link between obesity and prostate cancer death that could lead to new treatment and prevention strategies.

Patient Summary: Obese men are at increased risk of advanced prostate cancer and prostate cancer death, but the mechanisms are not known. We found different gene expression patterns in prostate tumors of very overweight/obese men, some of which were related to expression of genes involved in chromatin remodeling, a major mechanism of gene regulation often disrupted in cancer cells. Higher expression of chromatin-related genes was associated with more aggressive disease and worse outcomes, giving clues to the underlying biology of obesity and prostate cancer and providing possible strategies for prevention and treatment.

Introduction

Identification of risk factors that drive progression in prostate cancer has been a challenge. Obesity is a modifiable risk factor linked to advanced disease and worse cancer-specific outcomes among prostate cancer patients [1, 2]. Given high rates of obesity, an understanding of the relationship between excess body weight and worse prostate cancer outcomes has important clinical and public health implications. While several mechanisms have been proposed [3, 4], what drives the association between obesity and aggressive prostate cancer remains poorly understood.

In this study, we sought to explore the link between excess body weight and lethal prostate cancer using whole transcriptome gene expression profiles of prostate tissue. We assessed differences in gene expression in tumor and adjacent normal tissue according to prediagnosis body mass index (BMI) and examined the role of these genes in prostate cancer-specific mortality.

Material (Patients) and Methods

Study population

This study was nested among prostate cancer patients in the prospective Physicians' Health Study (PHS) and Health Professionals Follow-up Study (HPFS). PHS I and II began in 1982 and 1997 respectively as randomized primary prevention trials of aspirin and supplements among 29,067 U.S. physicians [5, 6]. HPFS is an ongoing cohort study of 51,529 U.S. health professionals followed since 1986 [7]. Both cohorts completed annual or biennial questionnaires on lifestyle and health. Incident prostate cancer was confirmed by review of medical records and pathology reports. The studies

were approved by institutional review boards at the Harvard T.H. Chan School of Public Health and Partners Health Care.

Following confirmation of diagnosis, archival formalin-fixed paraffin-embedded (FFPE) prostate tissue specimens from radical prostatectomy (RP) or transurethral resection of the prostate (TURP) were retrieved from treating hospitals. Gene expression profiling was performed on a subset of 402 of the 2,200 cases with available tissue diagnosed from 1982–2005, using an extreme case sampling design: 113 lethal cases (metastatic disease or prostate cancer death) and 289 indolent cases (survived ≥8 years after diagnosis without evidence of metastases). For 200 of these men we also profiled adjacent normal tissue.

Gene expression profiling

To measure gene expression in archival FFPE tissue specimens, whole-transcriptome amplification using the WT-Ovation FFPE System V2 (NuGEN) was paired with microarray technologies using the GeneChip Human Gene 1.0 ST microarray (Affymetrix) as previously described [8, 9]. Expression profiles were processed by regressing out technical variables including mRNA concentration, block age, batch (96-well plate), percentage of probes above background, log-transformed average background signal, and median of the perfect match probes for each probe intensity of the raw data. The residuals were shifted to the original mean expression values and normalized using the robust multi-array average method [10, 11]. We mapped gene names to Affymetrix transcript cluster IDs using the NetAffx annotations as implemented in Bioconductor annotation package pd.hugene.1.0.st.v1, resulting in 20,254 unique

gene names. Gene expression data are available through Gene Expression Omnibus (GSE79021).

Anthropometric data

BMI was calculated using height and weight reported on questionnaires immediately preceding cancer diagnosis. In HPFS, self-reported measurements of weight show high validity [12]. The mean prediagnosis BMI was 25.4 kg/m^2 (range 19.0-36.8 kg/m^2) and the mean time between BMI measurement and prostate cancer diagnosis was 1.3 years (range 0-11.3 years). Because the number of men in our study above the World Health Organization cut-off for obesity (BMI ≥30 kg/m^2) was low (N = 27), we divided BMI into the following categories for subsequent analyses: 18.5 to <25 (healthy weight), 25 to <27.5 (overweight), and ≥27.5 kg/m^2 (very overweight/obese), with a sensitivity analysis using BMI ≥30 for the top category.

Clinical and follow-up data

Information about age and date of diagnosis, prostate-specific antigen (PSA) level at diagnosis, and clinical and pathologic stage was abstracted from medical records and pathology reports. Study pathologists provided a standardized histopathologic review of each case including Gleason grading. Information on the development of metastatic disease was collected through follow-up questionnaires. Review of medical records and death certificates were used to determine date and cause of death. Lethal prostate cancer was defined as distant metastases or prostate cancer-specific death with follow-up through March 2011 (PHS) or December 2011 (HPFS).

155

156 **Statistical analysis**

157 Linear regression as implemented in the Bioconductor package *limma* was used to
158 assess differential expression of individual genes by BMI [13]. Gene Set Enrichment
159 Analysis (GSEA) [14] was performed to identify the association between BMI and
160 expression of 589 Gene Ontology (GO) Biological Process gene sets from the
161 Molecular Signature Database v4.0, using software from the Broad Institute
162 (http://software.broadinstitute.org/gsea/index.jsp). Genes were ranked based on a
163 signal-to-noise metric comparing very overweight/obese (BMI ≥27.5 kg/m^2) to healthy
164 weight (BMI 18.5 to <25 kg/m^2) men. An Enrichment Score (ES) was calculated for each
165 gene set based on a weighted Kolmogorov-Smirnoff statistic and the top ranked genes
166 contributing to the ES were identified as the leading edge subset. Significance was
167 estimated using 10,000 phenotype-based permutations. The normalized enrichment
168 score (NES) and false discovery rate (FDR) were used to identify the top GO biological
169 processes differentially expressed by prediagnosis BMI status. Gene sets with FDR <
170 0.25 were considered for subsequent analyses. The Enrichment Map Cytoscape Plugin
171 [15] was used to visualize GSEA results as gene set networks.

172 To further explore the five chromatin-related gene sets identified in GSEA, we
173 created a “metagene” score representing chromatin gene expression by averaging the
174 normalized (mean centered, variance scaled) expression values of the leading edge
175 genes from these gene sets.

176 We used t-tests to compare mean scores between tumor and adjacent normal
177 tissue, and Pearson correlations to measure the relationship between the score and

BMI. We used logistic regression adjusted for age and year at diagnosis to calculate odds ratios (ORs) and 95% confidence intervals (CIs) for the association between the "metagene" score and lethal prostate cancer. P-values were from the Wald test. We adjusted for Gleason grade to test whether the score independently predicted lethal cancer.

Finally, we used logistic regression to evaluate whether the "metagene" score mediated the association between BMI and lethal prostate cancer, adjusting for age, date at diagnosis, and Gleason grade. We used mediation analysis to calculate the percentage of the association between BMI and lethal prostate cancer explained by the score [16].

Mediation analyses were performed using SAS version 9.3. R version 3.1.0 was used for all other analyses. All statistical tests were two-sided, with p-values < 0.05 considered statistically significant.

Results

Table 1 describes the clinical characteristics of the study population according to prediagnosis BMI. Among 402 men, 192 (47.8%) were healthy weight (BMI 18.5 to <25 kg/m^2), 126 (31.3%) were overweight (25 to <27.5 kg/m^2), and 84 (20.9%) were very overweight/obese (BMI ≥27.5 kg/m^2) prior to prostate cancer diagnosis. No statistically significant differences were observed for clinical characteristics across BMI categories. However, there was a suggestion of increased pathologic TNM stage with increasing BMI.

Gene sets enriched in prostate tissue of overweight and obese prostate cancer patients

We compared gene expression in the highest and lowest BMI categories. No individual genes were significantly differentially expressed by BMI in tumor or adjacent normal tissue after adjusting for multiple comparisons (data not shown).

GSEA [14] identified fifteen gene sets upregulated and two gene sets downregulated in the tumor tissue of very overweight/obese vs. healthy weight patients with FDR < 0.25 (Figure 1, Tables S1, S2). Among these top results, there were several networks of overlapping gene sets involved in chromatin regulation, RNA processing, and cellular disassembly (Figure 2). These pathways were not differentially expressed in adjacent normal tissue, suggesting the results are tumor-specific (Tables S3, S4). To address differences in sample sizes for tumor and adjacent normal tissue, we repeated the GSEA on the subset of tumor samples that also had normal tissue data and found that 9 of 15 upregulated gene sets from the full analysis remained enriched at FDR < 0.25 (Tables S5 and S6).

Characterization of chromatin gene set network

Five of the 15 gene sets enriched in tumor tissue of very overweight/obese patients included chromatin modification and remodeling genes involved in regulation of chromatin structure and function (Figure 2). All five of these chromatin-related gene set were also ranked in the top ten in a sensitivity analysis using 30 kg/m^2 as the cutoff for the high BMI group (data not shown).

Given the importance of epigenetics in cancer development and progression [17], we further characterized this network by creating a "metagene" score based on expression levels of the 35 genes in the chromatin gene set network that comprised the GSEA leading edge subset (Table 2). This "chromatin gene score" was greater in tumor tissue than in adjacent normal tissue (p-value = $2x10^{-4}$). As expected, the "chromatin gene score" was positively associated with prediagnosis BMI in tumor tissue (p-value = $6x10^{-5}$) but not in adjacent normal tissue (p-value = 0.46).

Table 3 illustrates the clinical characteristics of the cohort according to tumor "chromatin gene score". The score was significantly positively associated with Gleason grade >7 (chi-square trend test p-value = $3x10^{-4}$). It was positively, but not significantly, associated with pathologic stage T3/T4 disease (chi-square trend test p-value = 0.11).

Chromatin gene expression and lethal prostate cancer

The tumor "chromatin gene score" was positively associated with risk of lethal prostate cancer, with an OR of 6.78 (95% CI = 3.42-14.16) comparing extreme quartiles of the score. With adjustment for Gleason grade, the OR for lethal prostate cancer was only slightly attenuated (OR = 5.01, 95% CI = 2.31-11.38) (Table 4). Adjustment for BMI did not alter these associations (results not shown).

BMI, chromatin gene expression, and lethal prostate cancer

To explore whether chromatin modification and remodeling mediates the relationship between excess body weight and lethal prostate cancer, we assessed the association between BMI and lethal cancer with and without adjustment for tumor "chromatin gene

score". Per 5-unit increase in prediagnosis BMI, the OR for lethal prostate cancer was 1.70 (95% CI = 1.16-2.53). Adjustment for chromatin score reduced this OR to 1.41 (95% CI = 0.94-2.12). In a mediation analysis, 36% of the BMI–lethal prostate cancer link was explained by the "chromatin gene score". Adjustment for Gleason grade did not affect these results (results not shown).

Discussion

There is compelling evidence linking obesity to aggressive prostate cancer, but the biology underlying this relationship is unclear. We found several networks of gene sets involved in chromatin regulation, RNA processing, and cellular disassembly enriched in the tumor tissue of overweight and obese prostate cancer patients compared to those of healthy weight. Focusing on chromatin regulation-related gene sets, we found that tumors with high expression of these genes had higher Gleason grades and were at increased risk of lethal prostate cancer, independent of grade. This suggests that obesity may promote tumor progression in part by influencing the epigenetic state of prostate cancer.

Epigenetic alterations are a common feature of cancer and are emerging as important drivers of tumor progression [17]. In prostate cancer, DNA methylation has been linked to metastatic disease [18]. In addition, extensive remodeling of the histone code occurs in prostate cancer and, in cooperation with DNA methylation, results in transcription of key oncogenes, microRNAs, and cancer biomarkers [19]. The current analysis identified genes encoding chromatin remodeling factors and histone modification enzymes, including histone deacetylases (HDACs). These mechanisms

work together to regulate gene transcription as well as other cellular processes including DNA replication and DNA damage repair [20]. HDAC overexpression in prostate cancer specimens has been linked to adverse tissue features and worse outcomes [21]. Furthermore, global histone modification patterns have been correlated with recurrence [22].

Epigenetic regulation mediates the reversible effects of environmental exposures and lifestyle factors on carcinogenesis and tumor progression [23]. Observational and experimental studies have begun to provide evidence for epigenetic alterations related to obesity; however, most human studies in this area were conducted in blood or adipose rather than tumor tissue and have focused on DNA methylation [24]. Our novel findings suggest that obesity impacts epigenetic regulation in prostate tumor tissue through chromatin-related processes.

Interestingly, our analysis of normal tissue found no association between BMI and chromatin-related gene expression, suggesting that characteristics specific to tumor tissue may render susceptibility to the effects of excess body weight. Along these lines, our group previously demonstrated that obesity is linked to worse prognosis among men with tumors harboring the *TMPRSS2:ERG* gene fusion [25], supporting the idea that obesity interacts specifically with certain molecular features of prostate cancer to drive tumor progression. Further investigation is needed to determine what role such tissue factors play in the epigenomic rewiring observed in overweight and obese patients.

Our study is the largest to date to evaluate prostate cancer gene expression signatures of patients with high BMI and the first to relate such signatures to disease outcomes. One study of 12 patients evaluated gene expression profiles of prostate

tumor and matching normal tissue according to BMI at treatment and found an association of BMI with altered expression of lipid metabolism and cholesterol homeostasis genes [26]. A second study focused on gene expression in periprostatic adipose tissue by BMI among 18 prostatectomy patients [27]. These authors found altered expression of genes involved in adipogenic/antilipolytic, proliferative/anti-apoptotic, and mild immunoinflammatory processes in obese subjects.

Strengths of our study include its prospective design, well-characterized data on clinical and pathologic measures, including re-review of Gleason score, and long-term follow-up allowing for the study of lethal prostate cancer as the outcome.

Due to the lack of public data sources with both gene expression and BMI data, we were unable to validate our results in an independent cohort. Thus, additional studies are needed to confirm these findings. The cohort is almost exclusively white men, and our conclusions may not apply to men of other ethnic groups. A potential limitation of the study is the use of BMI as an imperfect measure of obesity; however, BMI is the most widely used method for assessing adiposity in epidemiologic studies, and its correlation with obesity-related biomarkers is comparable to more direct measures of body fatness [28].

We cannot completely rule out that obesity affects prostate cancer outcomes at least in part through its effect on detection and treatment, rather than through true biological differences in tumors themselves [29]. To address PSA detection bias, Ma *et al.* tested the association between BMI and prostate cancer mortality in the PHS cohort separately by pre-PSA and PSA screening eras and noted that the association remained largely unchanged [30]. While obese patients may receive different treatments

than non-obese patients [29], our study includes primarily men who underwent prostatectomy as curative treatment, which limits the possible impacts of treatment differences that are observed in the overall patient population.

Conclusions

This analysis provides the first comprehensive look at BMI-associated gene expression alterations in prostate tumor tissue. These results improve our understanding of the biology of aggressive prostate cancer and provide additional support for a causal relationship between excess body weight and prostate cancer survival. Many new epigenetic targets are emerging for the treatment of cancer. If confirmed, this study could provide insight into novel therapeutic targets that could augment lifestyle changes for men diagnosed with the disease.

Author contributions

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Analysis and interpretation of data: Ebot, Gerke, Labbe, Sinnott, Zadra, Rider, Tyekucheva, Wilson, Kelly, Shui, Loda, Kantoff, Finn, Vander Heiden, Brown, Giovannucci, Mucci

Drafting of the manuscript: Ebot, Mucci

Critical revision of the manuscript: All authors

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Obtaining funding: Loda, Giovannucci, Mucci

Administrative, technical, or material support: Finn

Supervision: Tyekucheva, Loda, Kantoff, Vander Heiden, Brown, Giovannucci, Mucci

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Figure Legends

Figure 1. Gene Ontology Biological Process gene sets enriched in tumor tissue of overweight/obese patients compared to healthy weight patients. Gene sets identified by Gene Set Enrichment Analysis with a false discovery rate less than 0.25 are shown. Gene Ontology terms are ordered according to the normalized enrichment signal. Numbers next to each bar represent the number of genes from the data set present in the particular biological process. Red bars represent upregulated gene sets and blue bars represent downregulated gene sets. NES = normalized enrichment score.

Figure 2. Enrichment Map of Gene Ontology Biological Process gene sets enriched in tumor tissue of overweight/obese patients compared to healthy weight patients. Gene sets identified by Gene Set Enrichment Analysis with a false discovery rate less than 0.25 are shown with an overlap coefficient cut-off of 0.5. Each gene set is a node and links represent gene overlap between sets. The larger the node the more genes in the gene set. Thicker lines represent more gene overlap between sets. Upregulated gene sets are in red and downregulated gene sets are in blue. Darker nodes represent more significant nominal p-values. The total number of genes in each gene set network is indicated.

476 **Table 1.** Characteristics of 402 men diagnosed with prostate cancer from 1982 to 2005 in the Health Professionals

477 Follow-up Study and the Physicians' Health Study according to prediagnosis body mass index

		Prediagnosis BMI		
Characteristic	**All men (N=402)**	**18.5 to <25.0 kg/m^2 (N=192)**	**25.0 to <27.5 kg/m^2 (N=126)**	**≥27.5 kg/m^2 (N=84)**
Year of diagnosis, N (%)				
Before 1990 (pre-PSA era)	45 (11.2)	27 (14.1)	10 (7.9)	8 (9.5)
1990-1993 (peri-PSA era)	112 (27.9)	54 (28.1)	36 (28.6)	22 (26.2)
After 1993 (PSA era)	245 (60.9)	111 (57.8)	80 (63.5)	54 (64.3)
PSA at diagnosis, ng/ml, median (Q1, Q3)[a]	7.3 (5.3, 11.6)	7.9 (5.6, 12.0)	6.2 (4.8, 11.5)	7.7 (5.5, 10.7)
Pathologic TNM stage, N (%)[b]				
T2 N0 M0	218 (59.4)	111 (62.7)	67 (58.8)	40 (52.6)
T3 N0 M0	129 (35.1)	54 (30.5)	43 (37.7)	32 (42.1)
T4/N1/M1	20 (5.4)	12 (6.8)	4 (3.5)	4 (5.3)
Clinical TNM stage, N (%)[c]				
T1/T2 N0 M0	349 (88.4)	168 (88.9)	111 (91.0)	70 (83.3)
T3 N0 M0	27 (6.8)	13 (6.9)	6 (4.9)	8 (9.5)
T4/N1/M1	19 (4.8)	8 (4.2)	5 (4.1)	6 (7.1)
Gleason grade, N (%)				
<7	57 (14.2)	29 (15.1)	17 (13.5)	11 (13.1)
3+4	138 (34.3)	67 (34.9)	45 (35.7)	26 (31.0)

4+3	102 (25.4)	45 (23.4)	33 (26.2)	24 (28.6)
>7	105 (26.1)	51 (26.6)	31 (24.6)	23 (27.4)
Tissue type, N (%)				
RP	368 (91.5)	177 (92.2)	115 (91.3)	76 (90.5)
TURP	34 (8.5)	15 (7.8)	11 (8.7)	8 (9.5)
Cohort, N (%)				
HPFS	254 (63.2)	124 (64.6)	77 (61.1)	53 (63.1)
PHS	148 (36.8)	68 (35.4)	49 (38.9)	31 (36.9)

[a]63 men missing PSA at diagnosis.

[b]35 men missing pathologic TNM stage.

[c]7 men missing clinical TNM stage.

SD = standard deviation; Q1 = lower quartile; Q3 = upper quartile.

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Table 2. Chromatin-related leading-edge genes identified by Gene Set Enrichment Analysis

Gene symbol	Gene name
ACTL6A	actin-like 6A
ARID1A	AT rich interactive domain 1A (SWI-like)
ASF1A	ASF1 anti-silencing function 1 homolog A (S. cerevisiae)
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3
CARM1	coactivator-associated arginine methyltransferase 1
CHAF1A	chromatin assembly factor 1, subunit A (p150)
HDAC2	histone deacetylase 2
HDAC3	histone deacetylase 3
HDAC8	histone deacetylase 8
HELLS	helicase, lymphoid-specific
HIRIP3	HIRA interacting protein 3
HMGB1	high-mobility group box 1
INO80	INO80 homolog (S. cerevisiae)
KAT2A	K(lysine) acetyltransferase 2A
KDM4A	lysine (K)-specific demethylase 4A
MTA2	metastasis associated 1 family, member 2
NAP1L1	nucleosome assembly protein 1-like 1
NAP1L2	nucleosome assembly protein 1-like 2
NAP1L4	nucleosome assembly protein 1-like 4
PBRM1	polybromo 1
RBBP4	retinoblastoma binding protein 4
RSF1	remodeling and spacing factor 1
SAFB	scaffold attachment factor B
SET	SET nuclear oncogene
SIRT1	sirtuin 1
SMARCA5	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5
SMARCC2	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily c, member 2
SUPT4H1	suppressor of Ty 4 homolog 1 (S. cerevisiae)
SUV39H2	suppressor of variegation 3-9 homolog 2 (Drosophila)
SYCP3	synaptonemal complex protein 3
TLK1	tousled-like kinase 1
TLK2	tousled-like kinase 2
TNP1	transition protein 1 (during histone to protamine replacement)
UBE2N	ubiquitin-conjugating enzyme E2N (UBC13 homolog, yeast)
WHSC1L1	Wolf-Hirschhorn syndrome candidate 1-like 1

482 **Table 3.** Characteristics of 402 men diagnosed with prostate cancer from 1982 to 2005 in the Health Professionals

483 Follow-up Study and the Physicians' Health Study according to the chromatin gene score

		Chromatin gene score			
Characteristic	**All men (N=402)**	**Quartile 1 (low) (N=101)**	**Quartile 2 (N=100)**	**Quartile 3 (N=100)**	**Quartile 4 (high) (N=101)**
Age at diagnosis, years, mean (SD)	65.7 (6.5)	66.7 (5.8)	65.0 (6.4)	64.5 (6.7)	66.5 (6.8)
Year of diagnosis, N (%)					
Before 1990 (pre-PSA era)	45 (11.2)	11 (10.9)	8 (8.0)	13 (13.0)	13 (12.9)
1990-1993 (peri-PSA era)	112 (27.9)	29 (28.7)	26 (26.0)	26 (26.0)	31 (30.7)
After 1993 (PSA era)	245 (60.9)	61 (60.4)	66 (66.0)	61 (61.0)	57 (56.4)
PSA at diagnosis, ng/ml, median (Q1, Q3)[a]	7.3 (5.3, 11.6)	6.7 (5.2, 13.4)	7.2 (5.2, 10.2)	7.3 (5.4, 11.0)	8.1 (5.8, 11.5)
Pathologic TNM stage, N (%)[b]					
T2 N0 M0	218 (59.4)	57 (61.3)	60 (63.8)	58 (63.0)	43 (48.9)
T3 N0 M0	129 (35.1)	32 (34.4)	29 (30.9)	28 (30.4)	40 (45.5)
T4/N1/M1	20 (5.4)	4 (4.3)	5 (5.3)	6 (6.5)	5 (5.7)
Clinical TNM stage, N (%)[c]					
T1/T2 N0 M0	349 (88.4)	90 (90.0)	87 (87.0)	87 (89.7)	85 (86.7)
T3 N0 M0	27 (6.8)	7 (7.0)	7 (7.0)	5 (5.2)	8 (8.2)
T4/N1/M1	19 (4.8)	3 (3.0)	6 (6.0)	5 (5.2)	5 (5.1)
Gleason grade, N (%)					
<7	57 (14.2)	20 (19.8)	13 (13.0)	15 (15.0)	9 (8.9)

3+4	138 (34.3)	39 (38.6)	45 (45.0)	36 (36.0)	18 (17.8)
4+3	102 (25.4)	25 (24.8)	18 (18.0)	26 (26.0)	33 (32.7)
>7	105 (26.1)	17 (16.8)	24 (24.0)	23 (23.0)	41 (40.6)
Tissue type, N (%)					
RP	368 (91.5)	93 (92.1)	95 (95.0)	92 (92.0)	88 (87.1)
TURP	34 (8.5)	8 (7.9)	5 (5.0)	8 (8.0)	13 (12.9)
Cohort, N (%)					
HPFS	254 (63.2)	54 (53.5)	70 (70.0)	61 (61.0)	69 (68.3)
PHS	148 (36.8)	47 (46.5)	30 (30.0)	39 (39.0)	32 (31.7)

[a]63 men missing PSA at diagnosis.

[b]35 men missing pathologic TNM stage.

[c]7 men missing clinical TNM stage.

SD = standard deviation; Q1 = lower quartile; Q3 = upper quartile.

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485 **Table 4.** Odds ratios and 95% confidence intervals for lethal prostate cancer according to the chromatin gene score

Chromatin gene score	N lethal events	OR (95% CI)[b]	P-value[a]	OR (95% CI)[c]	P-value[a]
Continuous, per 0.1 units	113	1.22 (1.14, 1.31)	4×10^{-8}	1.18 (1.09, 1.28)	4×10^{-5}
Categorical					
Quartile 1 (low)	15	ref	1×10^{-7}	ref	8×10^{-5}
Quartile 2	23	2.13 (1.02, 4.57)		2.03 (0.88, 4.81)	
Quartile 3	25	2.25 (1.08, 4.82)		2.04 (0.89, 4.79)	
Quartile 4 (high)	50	6.78 (3.42, 14.16)		5.01 (2.31, 11.38)	

[a]Quartiles modeled as a continuous variable (quartile 1 = 0, quartile 2 = 1, quartile 3 = 2, quartile 4 = 3) to test for linear trend across categories.

[b]Adjusted for age and year at diagnosis (continuous).

[c]Additionally adjusted for Gleason grade (continuous: <7 = 0, 3+4 = 1, 4+3 = 2, >7 = 3).

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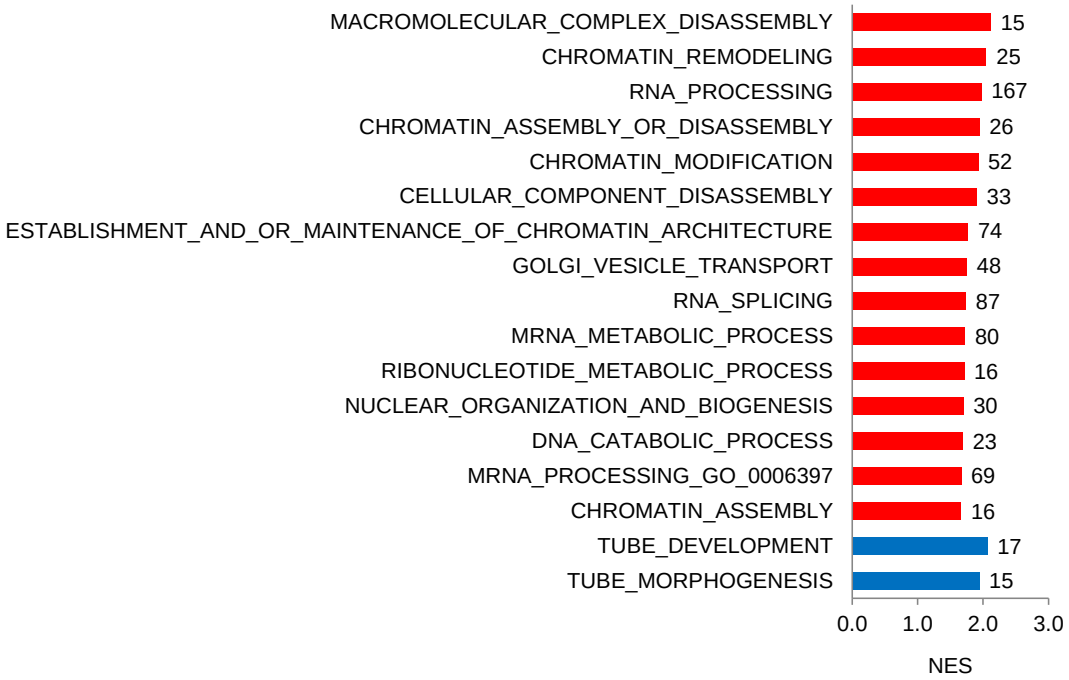
Figure 1


MACROMOLECULAR_COMPLEX_DISASSEMBLY
15
CHROMATIN_REMODELING
25
RNA_PROCESSING
167
CHROMATIN_ASSEMBLY_OR_DISASSEMBLY
26
CHROMATIN_MODIFICATION
52
CELLULAR_COMPONENT_DISASSEMBLY
33
ESTABLISHMENT_AND_OR_MAINTENANCE_OF_CHROMATIN_ARCHITECTURE
74
GOLGI_VESICLE_TRANSPORT
48
RNA_SPLICING
87
MRNA_METABOLIC_PROCESS
80
RIBONUCLEOTIDE_METABOLIC_PROCESS
16
NUCLEAR_ORGANIZATION_AND_BIOGENESIS
30
DNA_CATABOLIC_PROCESS
23
MRNA_PROCESSING_GO_0006397
69
CHROMATIN_ASSEMBLY
16
TUBE_DEVELOPMENT
17
TUBE_MORPHOGENESIS
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0.0
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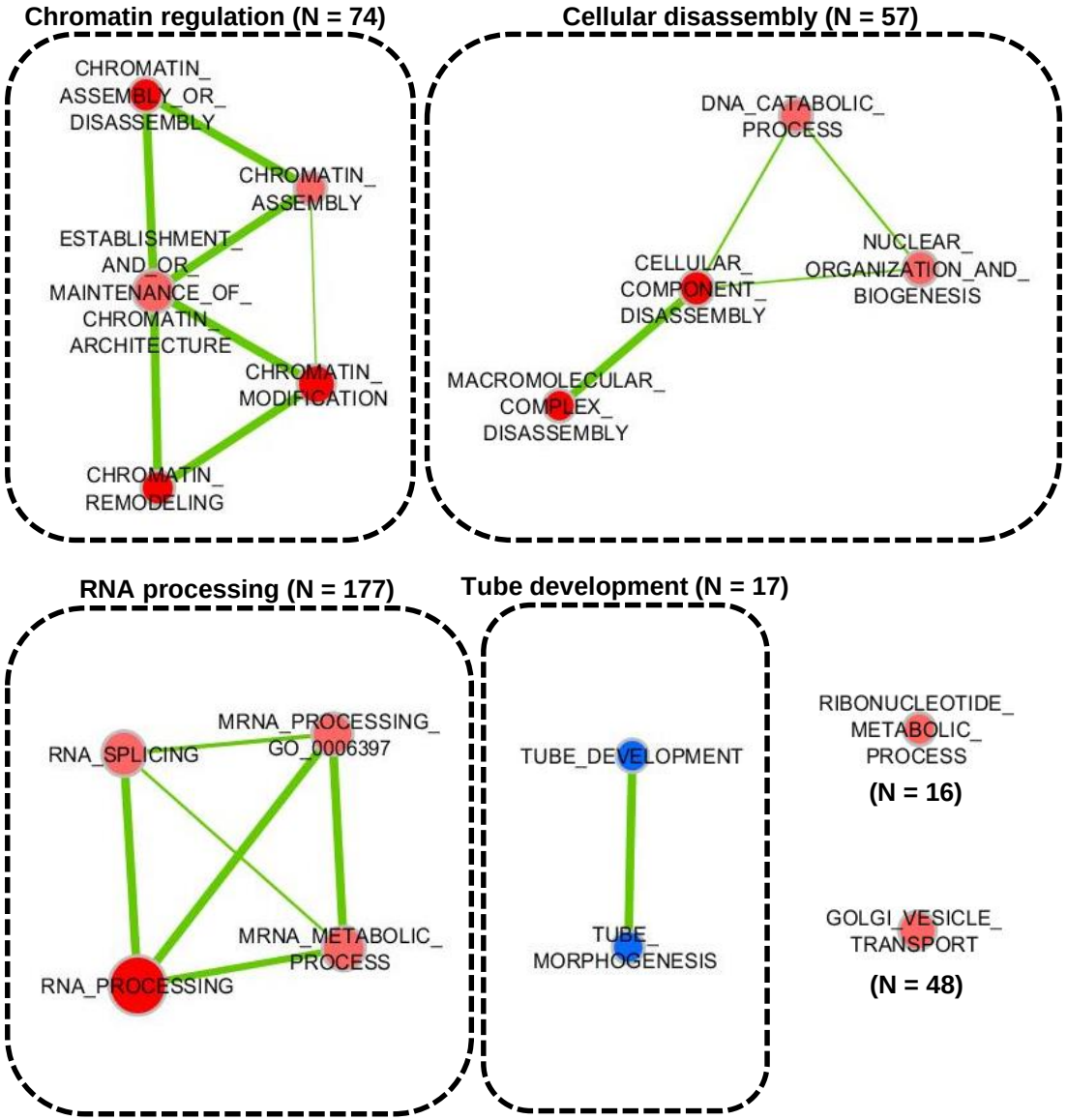
503 **Figure 2**



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REVIEW

Role of diet in prostate cancer: the epigenetic link

DP Labbé[1,2], G Zadra[1,3], EM Ebot[4], LA Mucci[4,5], PW Kantoff[1], M Loda[1,3] and M Brown[1,2]

Diet is hypothesized to be a critical environmentally related risk factor for prostate cancer (PCa) development, and specific diets and dietary components can also affect PCa progression; however, the mechanisms underlying these associations remain elusive. As for a maturing organism, PCa's epigenome is plastic and evolves from the pre-neoplastic to the metastatic stage. In particular, epigenetic remodeling relies on substrates or cofactors obtained from the diet. Here we review the evidence that bridges dietary modulation to alterations in the prostate epigenome. We propose that such diet-related effects offer a mechanistic link between the impact of different diets and the course of PCa development and progression.

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INTRODUCTION

In the United States, an estimated 233 000 new prostate cancer (PCa) cases will be diagnosed and 29 480 patients will die from PCa in 2014, making this disease the most commonly diagnosed cancer and the second leading cause of cancer-related death in American men.[1] In Europe, PCa is estimated to be the third leading cause of cancer-related death in men for 2014, behind lung and colorectal cancers.[2] There are a few confirmed risk factors for PCa incidence overall, of which age is the most important: PCa is uncommon before 50 years of age and is rarely lethal before 60 years. In fact, 70% of PCa-related deaths occur after age 75.[3] African ancestry and a positive family history are also among the risk factors associated with PCa, and now numerous genetic risk loci have been validated in multiple studies.

The incidence of PCa worldwide can vary by as much as 50-fold between low- and high-risk populations. The large disparity in PCa incidence between the Eastern and the Western hemispheres, a trend observed even before the adoption of prostate-specific antigen testing in developed countries,[4] points to a key role of environmental factors, such as diet, as an etiologic factor in this disease.[5,6] This association is further supported by observations from Japanese immigrants in Los Angeles County, in whom PCa rates are almost quadrupled compared with Japanese living in their homeland and almost match the incidence rate seen in California native residents.[7]

PCa is characterized by complex genomic alterations that are highly heterogeneous and vary greatly from patient to patient, as well as within the same tumor focus. Such disparities can be partly explained by an underlying genomic instability.[8] In addition, PCa has been described as an 'epigenome catastrophe', because various changes in DNA methylation patterns can be detected well before the cancer becomes invasive,[9] suggesting that epigenetic changes are pivotal events in tumor initiation.[10,11] Interestingly, diet can induce various epigenetic modifications that result in global alterations in chromatin packaging; such stable and heritable changes regulate the access of the transcriptional machinery to target genes, and thereby modulate gene expression profiles.[9,12]

Here we introduce some of the evidence that supports the thesis that diet impacts PCa initiation and progression, and examine the hypothesis that these diet-related effects are, in part, mediated by epigenomic alterations.

DIET AND PCa: THE EPIDEMIOLOGICAL EVIDENCE

The impact of diet on cancer growth was first described in landmark studies at the beginning of the 20th century by researchers such as Peyton Rous, who reported that some tumors have a delayed growth and retarded development when transplanted to previously underfed hosts, whereas other tumors are unaffected by the host's diet.[13] We now know that not all cancer types are equally sensitive to dietary modulation,[14] a phenotype that may be attributed in part to defined genetic alterations.[15]

An increasing number of epidemiological and molecular studies point to a link between diet and PCa, particularly for cancers that are more aggressive. Despite this, the role of specific dietary components in PCa development and progression is still unclear. In 2007, the World Cancer Research Fund/American Institute for Cancer Research reported that a diet rich in foods containing lycopene/cooked tomatoes or selenium (nota bene, selenium content in food is mirrored by the soil's selenium abundance) has a protective effect against PCa, whereas diets high in calcium have been associated with increased risk for PCa.[16]

Following this line of reasoning, the role of lycopene and tomato products in PCa prevention has been extensively studied and, although evidence is mixed, available data suggest an inverse association between increased consumption and PCa.[17] In the prospective Health Professionals Follow-up Study, consumption of tomato products was shown to be inversely associated with the incidence of total PCa as well as of advanced stage disease.[18] Also of interest, low levels of selenium have been associated with increased risk of PCa, particularly in relation to advanced or

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aggressive disease.[19] However, selenium supplementation did not significantly reduce the risk of developing PCa in the SELECT randomized trial, indicating that whether selenium intake is obtained directly from the diet or as supplements may impact differently PCa risk.[20] With limited evidence, other potential protective dietary elements include vitamin E, cruciferous vegetables, soy/isoflavones, polyphenols, fish/marine omega-3, coffee and vitamin D.[21–23] Conversely, a number of epidemiological studies have reported an increased risk of PCa for extreme categories of calcium intake,[24] with stronger associations for the risk of advanced or lethal disease.[18] The effect of folate intake (including folic acid supplementation) on PCa risk is conflicting. Although dietary and total folate intake is not associated with PCa risk, high circulating folate levels are associated with an increased risk of PCa,[25] a risk further heightened in patients of African ancestry.[26] With limited evidence, a high dietary intake of red meat and heterocyclic amines, saturated and monounsaturated fats, as well as the essential alpha-linolenic fatty acid (FA) promotes PCa development.[21,23]

FEEDING PCa

Evidence from preclinical models

The impact of diet on PCa progression has been evaluated in various mouse models (see the excellent review by Irshad and Abate-Shen[27] for a detailed overview of the strengths and limitations of each mouse model). It has been shown that a high-carbohydrate/high-fat diet enhances the growth of human PCa cell xenografts in mice.[28,29] In the Hi-Myc transgenic mouse model of PCa, a low-fat diet delays tumor progression,[30] whereas Hi-Myc mice maintained on a calorie-restricted diet display a reduced incidence of *in situ* adenocarcinoma compared with overweight controls (10% kcal from fat) or with mice on a diet-induced obesity regimen (60% kcal from fat).[31] Importantly, calorie-restricted mice do not develop invasive adenocarcinoma, and the frequency of invasive adenocarcinoma is significantly lower in mice fed a low-fat diet compared with mice on the diet-induced obesity regimen. Increased feeding of mice is correlated with greater activation of growth factor signaling,[31] and the greater frequency of prostate adenocarcinoma occurrence in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model has also been attributed to excessive calorie retention.[32] Moreover, a high-fat diet in LADY (12 T-10) transgenic mice is correlated with increased neuroendocrine differentiation, a marker of aggressive PCa.[33]

Similarly, $PTEN^{PE-/-}$ (PE, prostate epithelium) mice that are fed an omega-3 FA-rich diet display reduced PCa growth, slower histopathological progression and increased survival, whereas mice fed on an omega-6 FA-rich diet exhibit the opposite result. Insertion of an omega-3 desaturase (which converts omega-6 into omega-3 FA) into the $PTEN^{PE-/-}$ background rescues the phenotype of mice that are fed the high omega-6 diet.[34] Along the same lines, Yue *et al.*[35] recently observed that esterified cholesterol specifically accumulates in high-grade PCa and metastases, and that this accumulation results from the hyper-activation of the PI3K/AKT pathway following the loss of *PTEN*. Inhibiting acyl-coenzyme A (CoA):cholesterol acyltransferase (*ACAT-1*) results in a net depletion of stored cholesteryl ester, which impedes cell proliferation, migration and even tumor growth in murine xenograft models. Although the underlying mechanism responsible for this unforeseen phenotype, where cholesteryl ester fuels PCa growth, still remains to be fully defined,[35] these observations are further strengthened by the recent findings that ACAT-1 expression can serve as a prognostic marker that readily distinguishes indolent from aggressive PCa.[36]

The human data

In an elegant *ex vivo* study, Aronson *et al.*[37] randomized men with PCa (but not currently under treatment) to either a low-fat (15% kcal) high-fiber and soy-supplemented diet or a typical high-fat (40% kcal) Western diet for 4 weeks; they found that proliferation of LNCaP cells grown in a medium containing 10% human serum from these patients is significantly inhibited only in the presence of serum from men maintained on a low-fat diet for 4 weeks. Consistent with this, obesity is correlated with a lower risk of early stage PCa, as well as an elevated risk of aggressive PCa.[38] In a meta-analysis, Cao and Ma[6] reported that an elevated body mass index of 5 kg/m^2 is associated with a 20% higher PCa-specific mortality. Obesity dysregulates a number of key hormonal pathways and it has been proposed that lower sex hormone-binding globulin, adiponectin and higher insulin, growth hormone, insulin-like growth factor 1 (IGF-1) may also contribute to the development of high-grade tumors in obese patients. In particular, the growth hormone/IGF-1 pathway, known to have a role in the metabolic syndrome (that is, increased blood pressure, high blood sugar level, abnormal cholesterol levels, excess in waist body fat), is implicated in PCa progression.[39–44] Interestingly, high circulating IGF-1 levels are more strongly associated with low-grade than high-grade PCa. This result may reflect a greater dependency of differentiated neoplastic cell on circulating IGF-1 compared with undifferentiated cells that may be less responsive due to a constitutively active PI3K/AKT pathway.[45] In addition, among men diagnosed with PCa in the Physicians' Health Study, excess body weight and a high plasma concentration of C-peptide (a surrogate for insulin levels) both predispose men to an increased likelihood of dying of the disease, further suggesting a role for insulin in PCa progression in obese men.[46] Finally, men with hypercholesterolemia are also more at risk of developing aggressive PCa, a trend reverted by statins' intake.[47]

Collectively, these results obtained from preclinical models and human data demonstrate that both diet and obesity can alter PCa risk and progression. Obviously, the influence of these factors on PCa development is complex and involves a large number of 'classical' signaling pathways (reviewed by Venkateswaran and Klotz[48]). In this review, we propose that diet also alters the prostate epigenome and affects the course of the disease.

THE ALTERED EPIGENOME OF PCa

Epigenetic marks, including DNA methylation and histone modifications, are critical for maintaining a carefully regulated state for the cell. These marks affect local as well as global chromatin packaging, which in turn dictates the sets of active and inactive genes at any given time. It is now clear that cancer development is at least supported,[49] if not initiated,[11] by alterations of the epigenome, which then leads to transcriptional rewiring. Epigenetic modifications observed in PCa evolve throughout disease progression.

DNA methylation in eukaryotes is defined as methylation of the fifth carbon on cytosine residues in CpG dinucleotides (5-methylcytosine). These covalently added methyl groups project into the major groove of DNA and alter transcription.[50] In PCa, genome-wide DNA methylation of cytosine residues in CpG dinucleotides is greatly impaired as the disease progresses to a metastatic stage and leads to global hypomethylation,[51] which can enable the transcription of normally unexpressed proviral and retrotransposon repeats,[52,53] followed by disruption of nearby genes and a predisposition to genomic instability.[53,54] Specific promoter hypomethylation can also reactivate proto-oncogenes such as the urokinase-type plasminogen activator (*PLAU*),[55,56] the matrix metalloproteinase-2 (*MMP2*)[56] or the heparanase (*HPSE*),[57] known to be implicated in tumor invasion and metastasis. On the other hand, promoter hypermethylation and silencing of specific

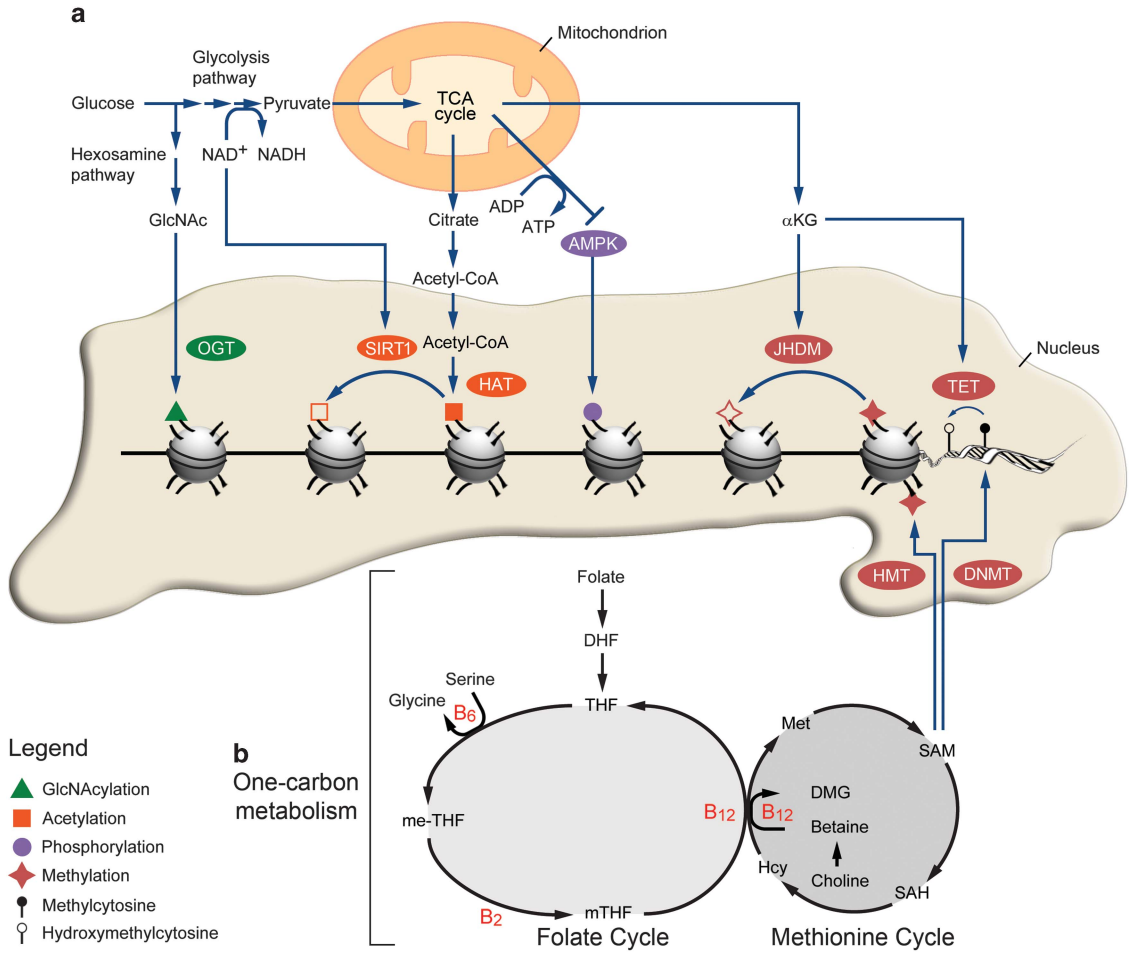

Figure 1. From metabolism to epigenetic remodeling. (**a**) SIRT1 activity depends on the NAD^+/NADH ratio modulated by glycolysis, while *O*-linked *N*-acetylglucosamine transferase uses GlcNAc produced by the hexosamine pathway. Pyruvate entering the tricarboxylic acid (TCA) cycle produces alpha-ketoglutarate, a critical cofactor for Jumonji domain-containing histone demethylase and TET. Acetyl-CoA is converted from the citrate generated by the TCA cycle and used as a donor by histone acetyltransferases. Finally, the increase in ATP/ADP ratio from the TCA cycle also inactivates AMPK. (**b**) SAM acts as a methyl donor for histone methyltransferases and TET and is obtained through the coordinate action of the folate and methionine cycles, termed one-carbon metabolism. αKG: Alpha-ketoglutarate; AMPK: 5′ AMP–activated protein kinase; ADP: adenosine diphosphate; ATP: adenosine triphosphate; B_2: vitamin B_2; B_6: vitamin B_6; B_{12}: vitamin B_{12}; DHF: dihydrofolate; DMG: dimethylglycine; DNMT: DNA methyltransferases; GlcNAc: N-acetylglucosamine; HAT: histone acetyltransferases; Hcy: homocystein; HMT: histone methyltransferases; JHDM: Jumonji domain-containing histone demethylase; OGT: O-linked N-acetylglucosamine transferase; me-THF: 5,10-methylenetetrahydrofolate; Met: methionine; mTHF: 5-methyltetrahydrofolate; NAD+: nicotinamide adenine dinucleotide (oxidized); NADH: nicotinamide adenine dinucleotide (reduced); SAH: S-adenosylhomocysteine; SAM: S-adenosylmethionine; SIRT1: sirtuin histone deacetylase 1; TCA: tricarboxylic acid; TET: ten eleven translocation; THF: tetrahydrofolate.

genes such as that for the detoxification enzyme *GSTP1* is observed in more than 75% of high-grade prostatic intraepithelial neoplasms and in almost all prostate carcinomas (95%),[58] and possibly sensitizes cells to DNA damage. In fact, hypermethylation of the *GSTP1* promoter is a highly specific PCa marker and is rarely detected in benign prostatic hyperplasia[58,59] and normal prostatic tissues.[59,60]

Global patterns of histone acetylation and methylation are also affected throughout PCa progression and can predict the risk of PCa recurrence.[61–63] Bert *et al.*[64] compared the long-range epigenetic remodeling that occurs in different PCa cell lines with that in normal primary cell lines. They used coordinate assessment of histone modifications, DNA methylation profiles and RNA expression; they identified 35 long-range epigenetic activation domains, each about 1 Mb long, and found that a total of 251 genes were activated within these domains—these include oncogenes and genes for microRNAs and PCa biomarkers (for example, *KLK3*, *PCA3*). In particular, alterations of histone marks in PCa cells were characterized either by an enrichment of active histone marks (H3K9ac and H3K4me3) or by the replacement of repressive marks (H3K27me3) by active marks (H3K9ac).[64]

This comprehensive analysis also revealed that, on a genome-wide scale, a subset of long-range epigenetic activation domains were not characterized by promoter hypomethylation, but rather by an extensive DNA hypermethylation in the CpG islands of promoter regions. On the basis of these findings, the authors propose that DNA hypermethylation of promoter regions can prevent the binding of transcriptional repressors, thereby facilitating transcriptional activity.[64] Their findings support a complex interaction between DNA methylation and the histone code in regulating gene transcription.

Together with the report that chromatin modifiers such as *CHD1*, *CHD5* and *HDAC9* are mutated in an important subset of primary PCa,[65] the above results demonstrate that the epigenome undergoes a complex and dynamic remodeling throughout disease progression.

EPIGENETIC MODIFICATIONS AND DIET

A fundamental feature of epigenetic remodeling is its reliance on substrates or cofactors obtained from the diet (Figure 1). When under situations of metabolic stress, the energy-sensing serine-threonine kinase 5′ AMP-activated protein kinase (AMPK) phosphorylates histone H2B at serine 36 and triggers a cell survival program.[66] Histone H2B is also targeted by an *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) residue on serine 112, a glucose-dependent modification that is often located near transcribed genes.[67] The activity of sirtuin histone deacetylase (SIRT) is dictated by the ratio of oxidized and reduced nicotinamide adenine dinucleotide (NAD^+/NADH), which can be modulated by fasting,[68] calorie restriction[69] or dietary supplementation of NAD^+ precursors.[70] Interestingly, in PCa, levels of both NAD^+ and GlcNAc metabolites are altered following seminal vesicle invasion or lymph node metastasis.[71] Alpha-ketoglutarate, an intermediate of the tricarboxylic acid cycle, is also a critical cofactor for histone demethylation by Jumonji domain-containing histone demethylase,[72] as well as for DNA demethylation by ten eleven translocation (Tet) proteins[73] (see the excellent review by Lu and Thompson[74] for details about these metabolite-dependent epigenetic modifications). In addition, the two most well-studied epigenetic processes, namely, methylation and acetylation, are also deeply connected to the diet.

Methylation: an epigenetic modification governed by one-carbon metabolism

DNA and histone methylation by DNA methyltransferases and histone methyltransferases, respectively, requires the transfer of a methyl group (catalyzed by a methyltransferase) from the methyl donor *S*-adenosylmethionine (SAM). Although DNA methylation is usually associated with transcriptional inhibition, the effect of histone methylation depends on the location of the methyl-lysine residue on the histone tail and also on the degree of methylation.[75] SAM is derived from methionine, an essential amino acid that can either be obtained from the diet *per se* or can be generated from homocysteine in a process that utilizes carbon derived from dietary folate, choline or betaine (also a product of choline oxydation) in a vitamin B12-dependent reaction.[76] This cyclic cellular process is termed one-carbon metabolism and is a bicyclic metabolic pathway that refers to the folate and methionine cycles (Figure 1). One-carbon metabolism integrates the donation of carbon units from nutrient inputs into essential cellular processes such as the regulation of redox balance, maintenance of the nucleotide pool, biosynthesis of proteins and the regulation of epigenetic modifications (reviewed by Locasale[77]). Erythrocyte levels of SAM can be altered by dietary intake of fat as well as of calories.[78] Evidence of a link between high serum levels of homocysteine (or deficiency in either folate or vitamin B12) and neural tube defects in the fetus during early stages of pregnancy led to mandatory worldwide folic acid fortification.[79] Finally, because one-carbon metabolism is central to cellular growth and proliferation, folate antagonists—first described in 1948 by Farber and Diamond[80] as a promising treatment for pediatric acute lymphoblastic leukemia—are also used as chemotherapeutic agents.

The yellow agouti (A^{vy}) mouse carries an intracisternal A particle (*IAP*) retrotransposon into the 5′ end of the *agouti* (*A*) gene and is a viable model for determining the impact of diet on epigenetic marks. When unmethylated and active, a cryptic promoter located within the 5′ end of *IAP*'s long terminal repeat hijacks the transcriptional control of the *agouti* gene and leads to ubiquitous expression of the agouti signaling protein; under normal conditions, this protein is restricted to hair cycle-specific patterns.[81] This yields mice that have a yellow coat color and develop multiple health issues such as type II diabetes, obesity and a higher frequency of tumor formation,[82] and serves as a phenotypic readout for a ready assessment of the methylation status of a promoter under different environmental conditions.

A major hallmark of the epigenome is its considerable plasticity during embryogenesis, which enables the differentiation of a single totipotent cell into more than 200 different cell types.[83] Wolff *et al.*[84] published a landmark study in which pregnant non-agouti (*a*/*a*) mothers mated with A^{vy}/*a* males were fed a methyl-supplemented diet (enriched in choline, betaine, folic acid, and vitamin B12), and found that fewer A^{vy}/*a* dams fed *in utero* with the methyl-supplemented diet had a yellow coat color and that this decrease was mirrored by an increased methylation of the A^{vy} proximal long terminal repeat.[85,86] In fact, the darkness of the coat color of the A^{vy}/*a* dams was directly correlated with the degree of methylation of the A^{vy} allele.[87]

In contrast, maternal exposure to bisphenol A (BPA) 2 weeks before mating and throughout gestation and lactation led to an increase in the proportion of A^{vy}/*a* dams that had a yellow coat color and carried a hypomethylated A^{vy} allele. This effect was negated when the BPA diet was supplemented with methyl donors.[88] Alternatively, peri-conceptional feeding of a methyl-deficient diet to female sheep resulted in adult offspring with CpG islands that were hypomethylated or unmethylated relative to animals fed on the control diet. Methyl-deficient diets also led to several health issues, ranging from higher body weight, increased fat, insulin resistance or elevated blood pressure in adult offspring.[89] Similarly, early peri-conceptional exposure to famine during the Dutch Hunger Winter in World War II led to hypomethylation of the imprinted*IGF2* gene in individuals compared with their same-sex siblings, a feature that was maintained for more than 60 years after the event itself.[90] Loss of *IGF2* imprinting is also a feature observed in PCa tissues,[91] as well as in proximal and distal tumor-associated tissues.[92]

Together, these results suggest that dietary modulation of rate-limiting factors of one-carbon metabolism generates long-lasting alterations in the methylation profile, and thus leads to phenotypic changes, in a given organism.

Histone acetylation is a nutrient-sensitive epigenetic mark

Acetylation of lysine residues on histones by histone acetyltransferases neutralizes the basic charge of the lysine, decreases electrostatic affinity between histone proteins and DNA and favors gene transcription via facilitated recruitment of the transcriptional machinery.[93] Lysine acetylation on proteins not only triggers gene transcription, but is also a critical posttranslational modification that regulates the activity of core metabolic enzymes.[94] Analysis of mass spectrometry data reveals that almost every enzyme involved in FA metabolism, glycogen metabolism, glycolysis, gluconeogenesis, the tricarboxylic acid cycle and the urea cycle is acetylated,[95] and functional analysis further documents a complex layer of regulation for protein lysine acetylation of metabolic enzymes. The acetylation status of these metabolic enzymes is responsive to environmental cues—such as the levels of amino acids, FAs or glucose—and modulates the activity and stability of the enzymes.[95]

Fluctuation in protein acetylation in response to dietary factors can be attributed, in part, to the availability of the acetyl group itself, which is obtained from the metabolite acetyl-CoA. Under nutrient-rich conditions, acetyl-CoA is generated by the ATP-citrate lyase (ACL), which catalyzes the conversion of citrate derived from the tricarboxylic acid cycle.[96] Alternatively, acetyl-CoA can be generated through the action of acetyl-CoA synthetases (ACECSs) from the pool of acetate, CoA and ATP. The activity of ACECSs is tightly regulated through reversible acetylation. Under low-nutrient conditions, the NAD^+/NADH ratio increases, activates SIRT1, which in turn de-acetylates and triggers ACECSs activity.[97] Therefore, the pool of acetyl-CoA, which is

governed by nutrient availability, controls the acetylation of metabolic enzymes as well as of histones at any given time.

Along these lines, studies in yeast reveal that levels of acetyl-CoA—which vary depending on the metabolic state—dictate cell growth, in part through the acetylation of histones at growth genes.[98] In yeast, this growth regulation mechanism may be balanced by the competition between histone acetylation and *de novo* FA biosynthesis for the same nucleocytosolic supply of acetyl-CoA, which normally matches growth signals with the required output in macromolecules.[99] In mammalian cells, histone acetylation is similarly dependent on the availability of acetyl-CoA, and inhibiting generation of acetyl-CoA through ACL knockdown thus results in global histone hypoacetylation.[96]

This critical mechanism for regulating cell growth is hijacked by the master transcription factor and proto-oncogene c-Myc, which is implicated in up to 70% of human cancers; Myc overexpression or deregulation results in cancer cells that become addicted to nutrients.[100] Specifically, Myc deregulation leads to the uptake of glucose and glutamine, which are carbon sources used to generate citrate (and consequently acetyl-CoA) through ACL activity.[101] Myc thus increases *de novo* FA biosynthesis and histone acetylation from glucose-derived acetyl groups.[102] Deregulation of cell metabolism by Myc leads to alteration of chromatin structure[103] combined with the generation of the biomass required for supporting uncontrolled cell growth.[104]

PCa: THE IMPACT OF DIET ON THE EPIGENOME

Several studies report a role for dietary components in the remodeling of the cancer epigenome (reviewed by Supic *et al.*[105]). In the context of PCa, the phytoestrogen genistein has the capability to partially demethylate CpG islands in the promoter region of specific genes such as *GSTP1*, leading to increased protein expression.[106] In PCa cell lines, genistein treatment also increases/restores expression of various tumor suppressors including *PTEN*, *p53*, *CYLD, p21WAF1/CIP1* and *p16INK4a*.[107,108] This feature is attributed to the coordinated demethylation and acetylation of H3K9 residues[107] or to increased expression of histone acetyltransferases that result in the enrichment of acetylated histones H3 and H4.[108] Similarly, the flavone apigenin also increases the acetylation of histones H3 and H4 *in vitro* and, when fed orally, significantly impedes PCa tumor growth *in vivo*. In this case, the phenotype is attributed to a marked reduction in histone deacetylase (HDAC) activity as well as in HDAC1 and HDAC3 protein expression.[109] Together, these results suggest that specific dietary molecules can alter PCa progression, in part by remodeling the epigenome. In addition, manipulating the content of dietary methyl donors or dietary fat alters the prostate epigenome and the course of the disease.

Dietary modulation of one-carbon metabolism to influence PCa development

As described above, one-carbon metabolism is central to DNA and histone methylation, as it generates SAM, the ultimate methyl donor. As in earlier studies with use of the A^{vy}/a model,[84] Shabbeer *et al.* used the Hi-Myc mouse model to investigate the impact of excess dietary methyl groups on PCa progression.[110] Overexpression of nuclear Myc protein is frequently detected in prostatic intraepithelial neoplasms, and in a majority of primary carcinomas and metastatic samples,[111] making the Hi-Myc mouse a particularly appropriate mouse model for the study of PCa. Mice were fed a control diet or a 'methyl' diet enriched in choline, betaine, folic acid, vitamin B12 and also in L-methionine and zinc sulfate while *in utero*[112] and during the first month of postnatal life, at which time all mice were fed the control diet. Although given only *in utero* and during early postnatal life, the methyl diet had a long-lasting effect on PCa development. At 5–7 months of age, no invasive adenocarcinoma was detected in prostates from Hi-Myc mice that were fed the methyl diet compared with a high incidence of invasive cancer in the control group. However, this difference in incidence was not observed in younger mice (at 3–5 months of age), suggesting that the methyl diet has an impact on the transition from mPIN to invasive adenocarcinoma, possibly via epigenomic changes.[112] These counterintuitive results indicate that timing might be critical in the context of modulating one-carbon metabolism, and can lead one to hypothesize that the methyl donor diet, if administered during the development of adenocarcinoma, would instead fuel uncontrolled tumor growth by maintaining a hyperactive one-carbon metabolism.

Along the same lines, Bistulfi *et al.* investigated the effects of manipulating dietary folate during disease progression in the TRAMP model, which relies on inactivation of pRb, p53 and PP2A following prostate-specific expression of SV40 large T and small t antigens.[113] TRAMP mice were fed one of three different diets at weaning: a folate-deficient diet, a folate-supplemented diet or a diet containing the recommended amount of folic acid for rodents.[114] Although folate supplementation had little to no effect on tumor growth, folate deficiency clearly improved PCa histopathological parameters compared with the control group, suggesting that folate might be a rate-limiting agent but only when it is under a certain threshold. Depletion of folate from the diet slowed the progression of cancer[114] and the robust arrest of disease progression was attributed by the authors to the secretory function of the prostate, which produces massive amounts of polyamines and exports them into reproductive fluids.[115] Indeed, no reduction in levels of polyamine was found in mice that were fed the folate-deficient diet, although polyamine synthesis draws on pools of SAM through the activity of SAM decarboxylase. This observation suggests that preferential use of SAM for polyamine synthesis under conditions of low folate in the prostate impedes other SAM-related pathways, such as the DNA methylation of CpG islands.[114] Consistent with this, a choline- and methionine-deficient diet led to increased expression of *Igf2* in the prostate of wild-type mice, a result that was mirrored by epigenetic changes at the gene promoter.[116]

In humans, the role of folate in PCa is unclear, although some evidence points to a positive association between high levels of circulating folate and PCa progression.[117] However, before considering the influence on the epigenome of dietary modulation of one-carbon metabolism, it is important to keep in mind that long-term deficiency of dietary methyl donors has important adverse effects. Folate depletion blocks *de novo* biosynthesis of thymidylate, leading to misincorporation of uracil into the DNA and culminating in single-strand DNA breaks[118]—as a consequence, prolonged dietary deficiency of methyl donors in mice leads to the development of intestinal tumors,[119] liver tumors and even to spontaneous mortality.[116] Thus, further experiments aimed at determining the timing, length and extent of a dietary intervention, to effectively impact the course of the disease while keeping side effects to a minimum, are warranted.

The cross talk between lipids and the prostate epigenome

As discussed above, manipulating dietary fat alters the progression of PCa in animal models. In 2010, Llaverias *et al.*[120] showed that increasing both dietary fat and dietary cholesterol significantly accelerates tumor progression in the TRAMP model, but the issue of whether cholesterol *per se* has a role in this aggravated phenotype was left unresolved. Pommier *et al.*[121] attempted to deconvolute these results using a mouse with a double knockout of the genes for the Liver X receptors alpha and beta ($Lxr\alpha\beta^{-/-}$), which encode nuclear receptors central to cholesterol homeostasis. The dorsal prostate lobes of $Lxr\alpha\beta^{-/-}$ mice fed on a standard diet were histologically similar to those of wild-type mice. But when $Lxr\alpha\beta^{-/-}$ mice were fed a high-cholesterol diet,

they accumulated intra-prostatic cholesteryl ester associated with mPIN development; gene expression analysis revealed that two prostatic tumor suppressor genes, *Nkx3.1* and *Msmb*, were downregulated in these mice. This event was attributed to an increase in the H3K27me3 mark at *Nkx3.1* and *Msmb* promoters, possibly a consequence of upregulation of the well-known prostate oncogene histone methyltransferase *Ezh2*.[121,122] Both *LXRβ* downregulation and *EZH2* upregulation have also been reported in human PCa.[123,124] Together with the recent report of abnormal cholesteryl ester accumulation in primary and metastatic human PCa (probably as a consequence PI3K/AKT hyperactivation following *PTEN*-loss),[35] these findings support a role for dietary cholesterol in influencing the prostate epigenome as well as disease progression of PCa.

Aside from dietary cholesterol, *de novo* lipid synthesis may also contribute to the regulation of epigenetic marks, especially histone acetylation. Indeed, *de novo* lipid synthesis is an important hallmark of PCa and correlates with tumor progression and poorer prognosis.[125] Use of an AMPK activator to block *de novo* lipogenesis impedes PCa growth and has been described as a promising treatment avenue, with or without the combined use of AR antagonists.[126] Along these lines, Kee *et al.* demonstrated that overexpression of the enzyme spermidine/spermine N^1-acetyltransferase (SSAT) leads to the diversion of pools of nucleocytosolic acetyl-CoA to polyamine catabolism. In the TRAMP model, overexpression of SSAT leads to a 70% decrease in the availability of acetyl-CoA and resulted in a genitourinary tract that is four times smaller than in control TRAMP mice.[127] It is thus tempting to speculate that *de novo* lipid synthesis observed in PCa also supports cell growth, in part, through global acetylation reprograming.[128]

CONCLUSIONS AND FUTURE DIRECTIONS

Mounting evidence implicates specific diets and dietary components in affecting the course of PCa and the risk of developing the disease. As PCa is considered to be an 'epigenetic catastrophe'[9] and because epigenetic marks rely on substrates or cofactors that are obtained from the diet, we suggest that the impact of diet on PCa development is, at least in part, linked to epigenomic remodeling.

Despite the promising results described here, a number of critical elements remain to be experimentally validated before the causality between diet and the prostate epigenome is established; these include the generation of a comprehensive epigenomic map of both healthy and neoplastic prostatic tissues from different models that are fed on controlled diets, and the metabolomics profile of matching tissues. Such an undertaking would facilitate the determination of the strength of the relationship between diet and the prostate's epigenome. Importantly, results obtained from PCa models should be carefully interpreted relative to their respective oncogenic drivers. Indeed, integrative metabolomic analysis recently revealed that PCa models driven by AKT1 are associated with the accumulation of aerobic glycolysis metabolites, while on the other hand MYC-driven PCa models are associated with dysregulated lipid metabolism.[129] Also, with the emergence of epigenetic-based PCa biomarkers (reviewed by Valdés-Mora and Clark[130]), the identification of common dietary- and cancer-dependent epigenetic alterations could be useful for patient risk stratification as well as for the development of specific dietary guidelines for defined patients.

Recently, epigenetic inhibitors that target DNA methyltransferases (azacitidine, decitabine) or HDAC (vorinostat, romidepsin) have been tested in clinical trials and approved by the US Food and Drug Administration for use in treating defined cancers.[131] Thus, deconvoluting the specific role of diet in rewiring the prostate's transcriptional network may yield critical information and may uncover dietary-related epigenetic pathways that can be therapeutically targeted to prevent or treat PCa.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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